ISTANBUL TECHNICAL UNIVERSITY★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

MOLECULAR CHARACTERIZATION OF A BORON-RESISTANT YEAST MUTANT OBTAINED BY EVOLUTIONARY ENGINEERING

M.Sc. THESIS

Musa TARTIK

Department of Advanced Technologies

Molecular Biology- Genetics and Biotechnology Programme

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

EVRİMSEL MÜHENDİSLİK ile ELDE EDİLMİŞ BOR STRESİNE DİRENÇLİ MUTANT MAYANIN MOLEKÜLER KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

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Musa Tartık, a **M.Sc.** student of ITU **Graduate School of Science, Engineering and Technology** student ID **521111118** successfully defended the **thesis** entitled **"Molecular characterization of a boron-resistant yeast mutant obtained by evolutionary engineering"**, which he prepared after fulfilling the requirements specified in the associated legislations,before the jury whose signatures are below.

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To my family;

FOREWORD

I would like to thank to God to give me the chance to meet my supervisor, Prof. Dr. Zeynep Petek Çakar. She was very helpful and encouraging for my thesis study; moreover, she was also very indulgent through my thesis period because of my job status. I am also very lucky to work in Yeast Lab and thank to my supervisor to give me the opportunity to work there.

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June 2013 Musa TARTIK

(Molecular Biologist)

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ABBREVIATIONS

cDNA : Complementary Deoxyribo Nucleic Acid
CDW : Cellular dry weight
DNA : Deoxyribo Nucleic Acid
EMS : Ethyl Methane Sulfonate
ESR : Environmental stress response
h : Hour
KAc : Potassium acetate
µg : Microgram
µL : Microliter
µm : Micrometer
mM : Millimolar
mg : Milligram
mL : Milliliter
min : Minute
OD : Optical Density
ORF : Open reading frame
PCR : Polymerase Chain Reaction
qPCR : Real-time polymerase chain reaction
RNA : Ribonucleic Acid
RPM : Revolution per minute
ROS : Reactive oxygen species
w/t : Wild Type
YMM : Yeast Minimal Medium
YPD : Yeast Extract- Peptone – Dextrose

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MOLECULAR CHARACTERIZATION OF A BORON-RESISTANT YEAST MUTANT OBTAINED BY EVOLUTIONARY ENGINEERING

SUMMARY

Boron is an essential micronutrient for plants and it is either necessary or beneficial for animals as an ultra trace element. It is also a growth supplement for *Saccharomyces cerevisiae*; however, increasing levels of boron (up to 80 mM) inhibit its growth.

The essentiality, utilization and toxicity of boron in living organisms are not fully understood yet. To date in the literature, three genes have been reported to be related to boron resistance in *S.cerevisiae; BOR1*, *ATR1* and *DUR3*. *BOR1* encodes a protein that is localized in the yeast plasma membrane. Moreover, Bor1p exports excess boron out of the cell to maintain ionic homeostasis inside the cell. The other gene, *ATR1* which was identified later, plays a major role to export boron out of the cell, when it is upregulated by high levels of boron. On the contrary, Dur3 transporter protein has a role against the efflux transporters Bor1 and Atr1. Although its functions are not clear, overexpression of *DUR3* increased boron levels inside the cells.

In this thesis work, the approach was to generate *S.cerevisiae* mutants with high boron resistance by using evolutionary engineering strategy that was previously applied successfully. Boron-resistant *S.cerevisiae* mutants were obtained and their phenotypic and physiological characteristics were determined. In order to identify the molecular mechanisms implicated in boron resistance, the whole transcriptomes of wild type and one of the most resistant mutants were compared via microarray analysis. Besides, *BOR1*, *ATR1* and *DUR3* expression profiles were also analysed by quantitative RT-PCR.

EVRİMSEL MÜHENDİSLİK ile ELDE EDİLMİŞ BOR STRESİNE DİRENÇLİ MUTANT MAYANIN MOLEKÜLER KARAKTERİZASYONU

ÖZET

Bor ve bileşik yapıları uzun zamandan beri bilinmesine rağmen teknolojideki yaygın kullanımı son zamanlarda yeni yeni artmıştır. Çevresel açıdan bulunurluğu zor olmasa da, maden rezervlerinin eşit dağılmadığı görülmektedir. Türkiye de bor madenleri açısından en zengin ülkelerden biridir. Yeryüzündeki yaşam formunun oluşumunda borun etkin rol aldığı düşünülmektedir. Bor elementi birçok organik bileşiğin yapısında yer almaktadır. Bu organik bileşikler, elektrofilik açıdan oldukça güçlüdür ve bunların en iyi bilinen fizyolojik formu borik asittir. Borik asit aynı yönde yerleşmiş hidroksil ve bazı amino gruplarını başlama kapasitesine sahiptir. Daha çok enzimlerin aktif bölgesinde ve 5 karbonlu ve furanoz halkası içeren karbonhidratlarda bulunan gruplarla bağ yaparlar. S-adenozil metyonin, nikotinamid adenin dinükleotidler, adenozin fosfatlar, diadenozin fosfat ailesi üyeleri ve RNA'ların 3' uçları gibi metabolizmada önemli moleküllerin ribozları borik asitle etkileşmeye açıktır.

Bor birçok bitki, hayvan ve mikroorganizmanın yapısına katılan önemli bir elementtir. Lakin her canlının denge sistemine dayanan yaşamsal aralığı yüksek miktarda bor elementinin toksik olarak algılar ve zarar görür. Toksisite mekanizması ve bor stres yanıtının organizmalardaki bileşenleri henüz açıklığa kavuşmamıştır. Bor direncinin moleküler mekanizması yönünde literatürde çok az bilgi bulunmakta ve mevcut bilgilerin çoğu son birkaç yılda yayınlanmış uluslararası makalelere dayanmaktadır. Bu nedenle bu konu, incelenmesi gereken güncel bir araştırma konusu olup, bu çalışmalar sonunda belirlenebilecek, bor direncine neden olan ve/veya bora bağlanabilen proteinlerin analizi ve bunların yapılarında meydana getirilebilecek değişiklikler ile borun kullanıldığı pek çok endüstriyel uygulamalarda biyobenzetim ve nanobiyoteknoloji yaklaşımı ile bu proteinler ve/veya yapıca onlara benzeyen hibrit moleküller kullanılarak proses verimleri arttırılabilir konuma gelecektir. Bu mekanizmaların deneysel olarak aydınlatılması ve anlaşılması model bir organizma kullanımını zorunlu kıllar. *S. cerevisiae* mayası, bu amaca hizmet edebilecek genetik niteliklere ve denenmişliğe sahip olmasının yanında bor stresi çalışmaları için uygun özelliklere sahip bir model organizmadır.

S. cerevisiae ökaryotik ve tek hücreli bir canlıdır. Bu özelikleri sayesinde genetik açıdan kolay manipüle edilebilirlik sağlarken, aynı zamanda diğer tüm ökaryotları temsil ederek onlar hakkında bilgi edinebilmek için model olmaktadır. Bu çalışmada model organizma olarak *S. cerevisiae* kullanılmıştır. Bu deneyde yaban tip *S. cerevisiae* dışında, evrimsel mühendislik yöntemiyle daha önce elde edilmiş olan bora dirençli mutantın fizyolojik ve moleküler karakterizasyonu yapılmıştır. Evrimsel müdendislik yönteminde hücreler başlangıç kültüründe kimyasal veya fiziksel yolla mutasyona ugratılırlar. Ardından, mutasyona uğradığı düşünülen hücre popülasyonu sürekli ve giderek artan stres faktörüne maruz bırakılır. Sürekli artan

stres ile popülasyon içinden istenilen stres koşuluna dayanıklı bireylerin seleksiyonu sağlanmış olur.

Bu deneyde stres faktörü olarak bor kullanılmıştır. En yüksek miktarda bor elementini tolere edebilen bireyler arasından en iyi bireyi seçmek için bor stresine ve yapılan çapraz direnç testlerine en fazla dayanan birey deneylerde kullanılacak mutant olarak seçilmiştir. Çapraz direnç testi; bireylerin ağır metaller, yüksek tuz, yüksek etanol gibi farklı stres koşullarına verdiği tepkiler, üreme durumlarını görmeye yarayan testlerdir.

Seçilen en iyi birey bor direnç mekanizmasında yabanıl tip ile karşılaştırmalı olarak incelenmiştir. Fenotipik analizler için katı besiyeri ortamına ekimler yapılarak mutant bireyin çapraz direnç mekanizmaları belirlenmiştir. Bu çalışma için farklı konsantrasyonda bor ve farklı stres koşullarına sahip (metal, iyon ve farklı bileşikler) katı besiyerleri hazırlanarak yabanıl tip ve mutant bireyin büyümeleri ve sonuçları gözlenmiştir.

Mutant birey ve yabanıl tip bor stresinin olduğu ve olmadığı sıvı kültürde üretilmiştir. Üreme sırasında üretilen ve tüketilen metabolitler HPLC (Yüksek Basınçlı Sıvı Kromotografi) ile ölçülmüştür.

Hem mutant hem yaban tipin farklı üreme koşullarında ve farklı zamanlarda, besi yerindeki glikoz tüketimi, maltoz, etanol, gliserol ve asetat üretimi belirlenmiştir. Böylelikle farklı zaman dilimlerinde iki suşta oluşan üretim ve tüketim farklılıklarına göre karşılaştırma yapılmıştır.

Ayrıca, enzimatik yöntemlerle hücrede oluşturulan trehaloz ve glikojen konsantrasyonları ölçülmüştür. Trehaloz ve glikojen *S. cerevisiae*'de stres durumunda üretilen metabolitlerdir. Trehaloz bir disakkarit, glikojen ise polisakkarittir. Hücrede rolü açık olarak bilinmemesine karşın, açlık ve stres durumuna karşı hücrenin depoladığı besin kaynakları olarak bilinir. Bunun yanında protein ve hücre membranı yıkımına karşı şaperonlar gibi işlev gördüğü de bilinmektedir. Bu sebeplerden ötürü mutantın ve yaban tipin trehaloz ve glikojen üretim seviyeleri belirlenmiş ve karşılaştırılmıştır. Aynı zamanda HPLC ile trehaloz ve glikojen deneylerinde suşlara bor stresi uygulanmış ve sonuçta stress faktörünün de bu metabolitlerin üretimi üzerindeki etkisi de gözlenmiş ve karşılaştırılmıştır.

Mutant birey ve yaban tipin moleküler karakterizasyonu için gen ekspresyon düzeyleri incelenmiş ve bu analiz için qPCR (quantitative PCR) ve mikroarray metotları kullanılmıştır. Yaban tipin ve mutantın, hem stresli koşulda hem de kontrol koşulunda, literatürde bor mekanizmasıyla ilişkili oldukları tespit edilen *BOR1*, *ATR1* ve *DUR3* genlerinin ekspresyon düzeyleri belirlenip kıyaslanmıştır.

BOR1 geninin bor elementinin hücre dışına çıkarılmasında efektif görev alan Bor1p zar proteinini kodladığı bilinmektedir. İlk defa bitki kök hücrelerinde AtBOR1 homoloğu olarak bulunan bu protein bor sıkıntısı çeken bitkilerin kök hücrelerine bor depolamasını sağlamakla birlikte ihtiyaç halinde köklerden yapraklara doğru bor taşınması için depo hücrelerinden borun atılmasında görev alan bir proteindir.

Son yıllarda keşfedilen *ATR1* geninin ise *BOR1* ile benzer olarak hücre dışına bor atımı yaptığı belirlenmiştir, ancak Atr1 zar protein hücre için toksik düzeye ulaşan bor miktarı azaltmak için boru dışarı atmaktadır.

DUR3 ise hücre içindeki bor ihtiyacını karşılamak için içeriye bor alımında görev alan Dur3p proteinin kodlar. Bu bilgiler doğrultusunda bor stresine dirençli mutant mayada bu genlerin ekspresyon düzeyleri incelenmiştir.

Tüm genom analizi yapılarak hangi yolakların stres sebebi ile nasıl değiştiği gözlemlenmiştir. Bu amaç doğrultusunda gen ekspresyon analizi için mikroarray yapılmıştır. Yaban tip ve mutantın tüm genom ekspresyon düzeyleri karşılaştırılmıştır. Mutantta ekspresyonu artan ve azalan genler belirlenmiş ve bu genlerin hangi yolaklarda işlev gördüğü araştırılmıştır. Bu sayede organizmadaki bütün genlerin transkripsiyon seviyeleri belirlenerek, organizmanın bor stresi durumunda hayatta kalmasını sağlayan yolaklar aydınlatılmıştır. Elde edilen bu sonuçların, önceki deneylerde açığa çıkarılan fizyolojik ve fenotipik sonuçlar ile desteklendiği gösterilmiştir. Mikroarray analizi sonuçları stres ortamında mutant bireyin enerji yolaklarını önemli ölçüde aktive ederek enerji üretiğini ortaya koymuştur. Ayrıca, mutantta, yüksek düzeyde enerji tüketimi gerektiren metabolik yolaklakların inaktif olduğu görülmüştür. Transkripsiyon ve protein sentezi bu yolaklardandır.

Özellikle, *S. cerevisiae*'de strese karşı direnç açısından önemli rol oynayan trehaloz ve glikojenin üretiminde rol oynayan genlerin ekspresyon düzeylerinde önemli ölçüde artış gözlenmiştir. Bu sonuçların ışığında bora dirençli mutant bireyde detaylı genetik ve proteom çalışmalarına devam edilmesi bor direnç mekanizmasının aydınlatılması açısından önem taşımaktadır.

1. INTRODUCTION

1.1 A Brief Introduction to *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is commonly known as baker's yeast or brewer's yeast. It is classified as a fungus since it has a chitin cell wall. However, it also known as an unicellular organism since it cannot form fruiting body, like other fungi. Classification of this organism is given in Figure 1.1 (Matthew *et al.*, 2004).

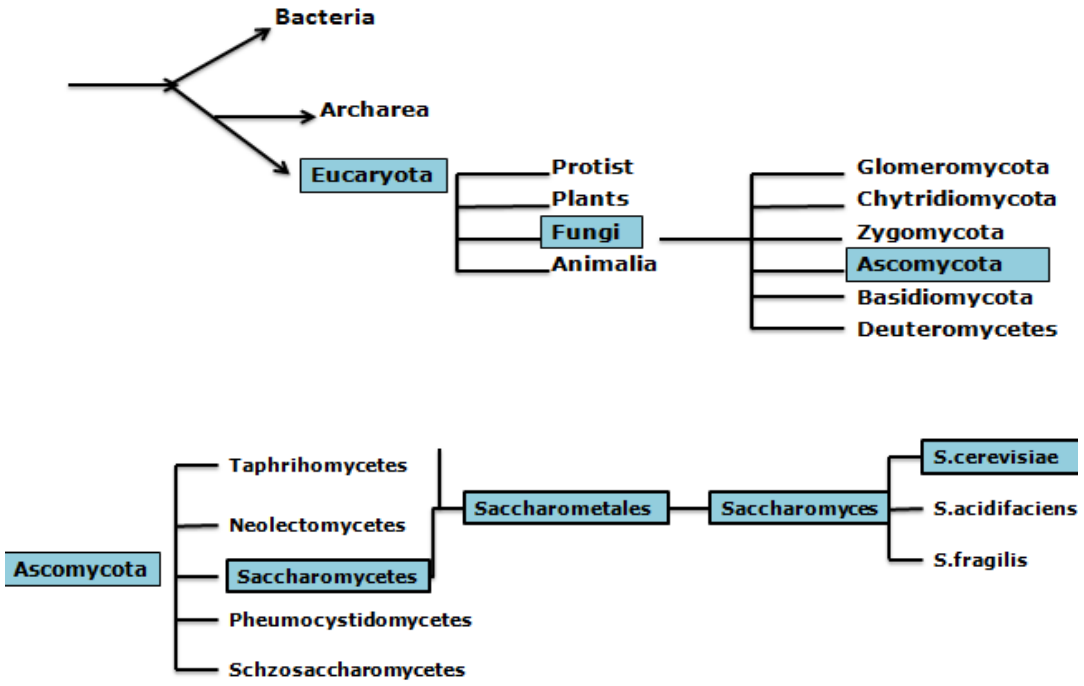

Figure 1.1: Classification of *S. cerevisiae*.

S. cerevisiae cells have oval shaped structure. Their cellular dimensions are around 10 µm long and 5 µm wide. They have thick, tough cell walls, without peptidoglycan layers. Lipid structures of their membrane are ester linked which is not very suitable for resistance under stress conditions (Alberts, 2002; Freeman *et al.*, 2005). Culturing of *S. cerevisiae* cells is accepted to be easy. They can be grown in both liquid and solid media with sufficient nutritional needs. They need a reduced carbon source (Acetate, glucose, glycerol etc.), a nitrogen source (such as ammonium sulphate, urea or various amino acids), vitamin biotin, salts and some metal elements for growth

(Alberts *et al.*, 2002). Doubling time of *S. cerevisiae* cultures varies between 90-140 minutes depending on the growth medium and strain type (Sherman, 1998).

S. cerevisiae is the first eukaryote to have its genome to be sequenced. It has 16 chromosomes. The total sequence of its chromosomal DNA, constituting 13.392 kb, was released in April, 1996. *S. cerevisiae* genome size is 1% of human genome size and is 3.5-fold of *E. coli* genome size. In contrast to the genomes of multi-cellular organisms, the yeast genome has a larger expressed region, which is 72% of the total sequence, consisting of 6275 genes. Control mechanisms are also similar to human cell control mechanisms, because of 23% of its genome overlap with that of human. Thus, the baker's yeast *S. cerevisiae*, being one of the earliest domesticated organisms, is also accepted as one of the best model eukaryotic microorganisms for biological studies (Erlend *et al.*, 2006; Geoffrey, 2000).

1.2 Life Cycle of *Saccharomyces cerevisiae*

Reproduction of *S. cerevisiae* could occur in two different ways, either vegetatively or sexually. Vegetative reproduction occurs by budding, which pinch out from the parent cell. Each parent cell produces almost 20-30 buds during its lifetime. Thus, the age of a parent can be detected by the number of the budscars on the cell surface (Egilmez and Jazwinski, 1989). A typical budding cell and the bud scars on the parent cell are shown in Figure 1.2.

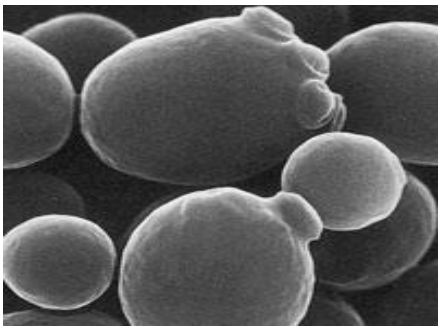

Figure 1.2: Scanning electron micrograph of budding yeast (Wheals, 2003).

Spindle pole body (SPB) carries signals for initiation of nucleus division that distribute chromosomes between mother and daughter bud cells. After separation, the daughter cell is always smaller than the mother cell. Although the body sizes are not equal, genetic material is distributed equally (Solomon, 1999; Lodish *et al.*, 1995).

Second way of reproduction is the sexual reproduction. There are two mating types in sexual reproduction of *S. cerevisiae* cells: *MATa* and *MATα,* which are formed as a result of the presence of one of the two alleles in *MAT* locus on chromosome III (Elion, 2000). *MATa* and *MATα* cells communicate with each other by secreting special pheromones and responds to the pheromones of the opposite type by forming a "shmoo" (Figure 1.3). Shmoos are distinguished by an elongated, pear-shaped and non-dividing structure in the cell culture. Vegetative cells can differentiate into shmoos only when the opposite mating type is present. The formation of the zygote with diploid nucleus is based on fusion of two shmoos. *S. cerevisiae* cells cannot divide indefinitely either in the haploid or the diploid state as well as higher plants and animals. In addition, transition between these states is possible (Alberts *et al.*, 2002). As a result of transition between two mating types, diploidization of haploid cultures may be observed.

Diploid yeast cells normally reproduce mitotically as a diploid, but when they are starved of nitrogen and in the presence of non-fermentable carbon sources, cells undergo meiosis and produce spores. A single cell can give rise to four spores encapsulated in an ascus. Spores are more resistant to environmental conditions than a normal cell. If the environmental conditions become available, spores germinate and commence growth as a haploid (Joseph-Strauss *et al.*, 2007).

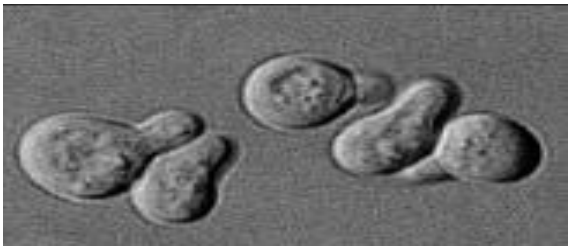

Figure 1.3: Shmoo formations in *S. cerevisiae* cells (Alberts, et al., 1998).

S. cerevisiae cells might either be heterothallic and homothallic. Heterothallic strains can either be in diploid or haploid forms, but homothallic strains are only found in diploid form (Figure 1.4).

Homothallic strains have an active HO gene that are capable of switching mating type, so they can change the genetic composition of the *MAT* locus to the opposite mating type (Madigan *et al.*, 2003).

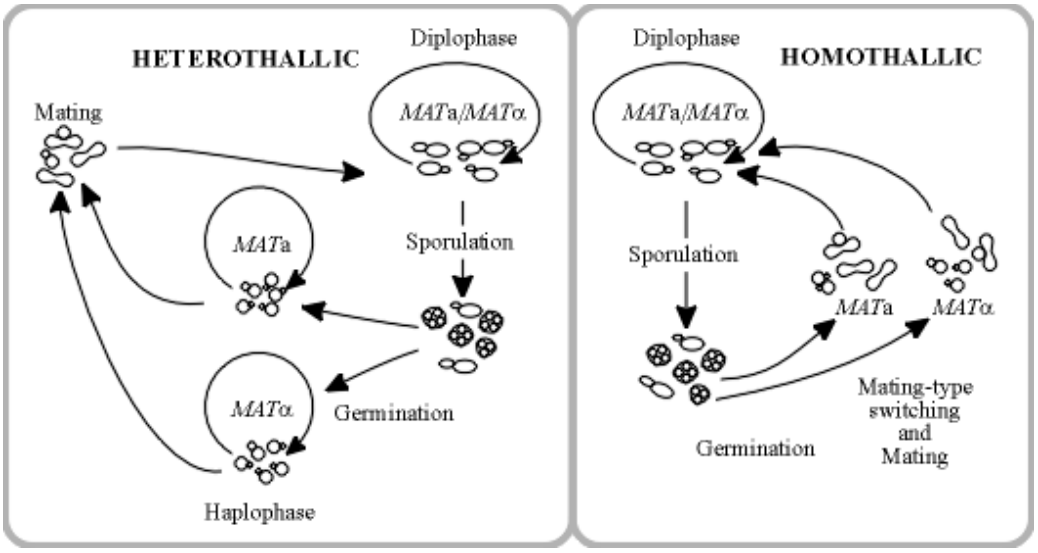

Figure 1.4: Reproduction cycles of heterothallic and homothallic strains of *S.cerevisiae* (Sherman F., 1998)

It was shown that diploid form of *S. cerevisiae* is relevant for evolution due to higher frequency of adaptive mutations than that of haploid form. Thus, it can be suggested that diploid cultures might have an evolutionary advantage over haploid ones (Paquin and Adams, 1983). The diploid cultures were shown to have 100-fold higher number of mutations as a result of high frequency of allelic crossover and gene conversion than in haploid cells (Ohnishi *et al.*, 2004). Perrot studied on diploid and haploid *S. cerevisiae* in both rich and poor medium to analyze growth differences and showed that diploid cultures have a global advantage over haploid cultures (Perrot, 1994).

1.3 Industrial Importance and Advantages of *S. cerevisiae*

Economically, yeast is significant for production of beer, wine, alcohol and bread since Ancient Egypt. In contrast to bacteria that can break down acids in alcohol to give wine a smoother taste, *S.cerevisiae* cells have the ability to perform primary fermentation reaction that converts glucose into ethanol. Lower alcohol inhibition is an advantage of *S. cerevisiae*, which makes it favorable for primary fermentation during industrial processes. It is known that it has the capability to produce up to 18% alcohol (Madigan *et al.*, 2003).

Yeast is also important for bread production to give a dough rise by producing CO_2 as leavening agent. During fermentation alcohol and carbon dioxide, which are produced by *S. cerevisiae* cells, give bread a lighter and finer texture (Black, 2002).

Use of *S. cerevisiae* since ancient times demonstrates that it is one of the most relevant organisms for mankind. Moreover, there are many other reasons that still draw scientists' attention towards *S. cerevisiae* and make it the most studied eukaryotic model organisms in molecular and cellular biology. First of all, it can be supplied either in haploid or diploid form and is commercially available. Under stress conditions, it produces ascospores by sporulation mechanism that diploid nucleus goes through meiosis producing four haploid nuclei which then incorporate into four stress-resistant ascospores, encapsulated in an ascus. Proliferation occurs when they are in haploid form, at which they can easily be isolated due to having dispersed cells and, therefore mutations can easily be studied (Snustad *et al.*, 2000).The other advantage is its short life cycle to produce new generations rapidly. Thus, culturing *S. cerevisiae* is less expensive and easier than other microorganisms. It is known to have GRAS (generally regarded as safe) status, so studying of *S. cerevisiae* requires only a few precautions. Therefore, these advantages make it attractive as a highly suitable system to experiment basic biological processes rather than many other higher eukaryotes including complex systems (Jaafar *et al.*, 2004).

As a result of all these advantages, *S. cerevisiae* is chosen as a model eukaryote as a reference to other higher eukaryotes. The genetic and biochemical knowledge about *S. cerevisiae* provide a privilege to be a model organism having a well-defined system.

1.4 Stress Responses of *S. cerevisiae*

All living organisms have/need stress response mechanisms to cope with different stress conditions. Because of that survival of cells critically improve their ability to sense challenges in the environment and to respond to new situations through the resistance mechanisms. It is known that yeast is one of the most suitable systems to study stress tolerance.

The consequences of various types of cellular stress mechanisms can be easily understood in yeast. There are many stress conditions studied in yeast; such as, heat, ethanol, hydrogen peroxide, rapid/slow freezing, salt, acetic acid, etc. (Lewis *et al.*, 1997).During the natural life cycle, there might be various continuous and unexpected biological and physical stress effects on yeast;

such as instability in temperature, osmolarity, and acidity of their environment, the presence of radiation and toxic chemicals, and long periods of nutrient starvation. Therefore, these harsh conditions have to be minimized by cellular stress signaling mechanisms to maintain organisms internal environment. For this goal, lots of stress response mechanisms rapidly evolved to compensate any perturbation in the surrounding environment (González-Párraga *et al.*, 2008).

Acording to Lewis and co-workers; yeast cells have cross-tolerance mechanism explained by contribution of existing stress resistance to adapt organism to other types of stress resistance (Lewis *et al.*, 1995). The findings reveal that *S. cerevisiae* has whole-genome stress response mechanism in which expressions of around 900 genes were altered under wide variety of environmental transitions. These genes were named as "Environmental Stress Response (ESR)", which is classified in two groups as repressed and induced genes. The first group of genes in ESR, about 600 genes, were repressed and downregulated that account for RNA metabolism, growth-related processes and protein synthesis. The other group consist of 300 induced genes to transcribe required metabolite for carbohydrate metabolism, protein folding and degradation, detoxification of reactive oxygen species (ROS), cell wall modification and DNA damage repair, etc. The stress response mechanism includes transcription factors Msn2p and Msn4p that are normally located in cytosol; however stressful conditions initiate translocation of transcription factors to the nucleus, where they bind to the stress response elements to keep under control upregulated genes in the ESR. Regulation of the induced genes, however, in ESR is not controlled by single system. There are different regulatory systems; many of them have not been clarified yet, activated under different environmental conditions (Gasch *et al.*, 2000).

1.4.1 Metal stress mechanisms of *S.cerevisiae*

All kind of organism composed of various chemical content of molecular structures that are assigned to different part of metabolism. The metals have also crucial role in each pathway inside this alive mechanism to integrate each organism to food chain. Hence, most of organisms have sufficient transport systems have been evolved for satisfying the requirement of metal ions. On the other hand, higher concentrations of metal can be threatening to cell survival (Eitinger and Mandrand-Berthelot, 2000).

Further investigations display that metal toxicity can be eliminated by organisms by production of uptake systems with a very low capacity, repression of transcription of the transporter genes in response to elevated metal concentrations, also activating a negative chemotactic response, and the expression of resistance determinants designed for metal expulsion from the cell (Eitinger and Mandrand-Berthelot, 2000).

Cell reactions against drastic stress conditions were divided into three stages (Figure 1.5). In the first point of view, the stress concentration that was applied disrupted plasma membrane much more than the initial stress tolerance of organism determined by the genetic make-up and history of the cell, so cells are killed immediately. On the other side, cells could repair the damage and alter their physiology and gene expression program which bears adaptation to the stress condition. Otherwise cells shuttle between metabolically active states without ability to grow mode which resulted to cell death. Lastly, only adaptation with physiological changes provides cells to grow again unless enough resources are available in the environment. However in the nutrient limited conditions, cells arrest at the START checkpoint that monitors nutrient availability. This situation causes growth rate decreases, which is lower than the cells grown in non-stressful conditions, of newly generated cells (Brul and Smits, 2005).

1.4.1.1 Brief information about boron

Boron is an essential micronutrient and metal. It is displayed in the Periodic Table with B letter and atomic number 5. In nature, boron could be found as two different isotope form; % 80 B^{11} and % 20 B^{10}, especially it can be included in minerals related to borax. Although it is 10^{-9} lower than hydrogen and 10^{-6} lesser than carbon, it spreads nearly lots part of earth (Lukaszewski, 2004).

Boron has two common allotrope rather than many types of other ones; Black crystalline boron and Brown amorphous boron (Figure 1.6). The semi conductor elemental boron is used to as a dopping agent in industry, on the other side boron composites are used as reactive during chemical synthesis, producing protectors and preparing insecticides (Kazanskii, 2002).

The first mention about property of boron inside biological plasma is formation of boric acid and lower concentration of borate anion $B(OH)^{-4}$.

These both forms of boron can react with various sugars, glycoproteins, glycolipids and phosphoinocitides that consist of hydroxyl groups to form of complexes.

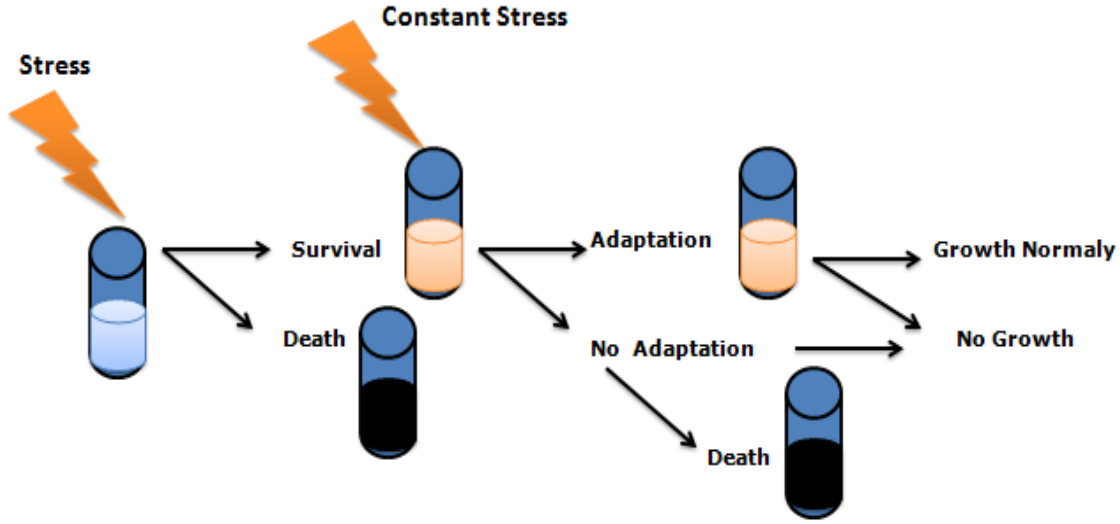

Figure 1.5: Schematic representation of the effects of stress on survival (Brul and Smits, 2005)

Because of these complex structures, it can be demonstrated that boron has a funtional role inside the cell membrane. Apart from these, it is known that the presence of boronophosphate at DNA structure increases stability of DNA (Lukaszewski, 2004).

The recent studies reveal that AI-2 autoinducer (Figure 1.7) that contained boron has crucial role as a signal molecule in quorum-sensing which is communication mechanism between microrganism. Moreover it is known that many species of bacteria can use quorum sensing to arrange their gene regulation by sensing of population density. AI-2 is also called Furonosil borate diester; and can be actively produced from reaction of 1-deoxy-3-dehydro-D-ribulose and boric acid in gram positive and negative bacteria. Besides, LuxS gene encoded AI-2 singal molecule is quite conserved among bacteria, thus it can inferred that AI-2 is a universal signal molecule (Coulthurst, 2002).

1.4.1.2. The necessity and toxicity of boron

It is believed that boron has an important function since early life stages of the evolution. Necessity criteria of boron are satisfying for at least some members of each phylogenetic kingdom.

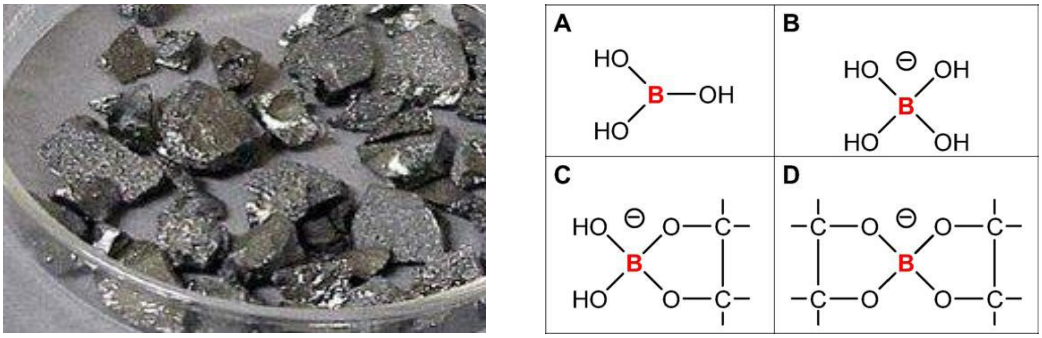

Figure 1.6: Black and Brown Boron (left side of the figure) and boric acid (A), borate anion (B) and chemical structure of ether formation of boron (C, D).

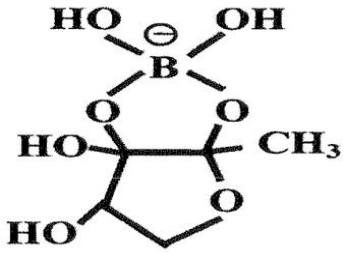

Figure 1.7: Bacterial Quorum-sensing molecules; autoinducer AI-2 (Lukaszewski, 2004).

These criteria involve:

1) Forming chalets or react with biological material

2) The presence in healthy tissues of organisms at comparable concentrations

3) Toxicity occurring only at high intakes

4) Short-term changes of tissue concentrations that occur after intake, maintenance by homeostatic mechanisms

5) Boron deficiency prevents the completion of the life cycle and growth

6) Physiologically important functions are impeded as a result of continuous boron depletion.

In 1923, a study about vascular plants was described that boron has an important role as an essential micronutrient (Warington, 1923). Then some types of studies show that boron has an essential role for microorganisms (Anderson and Jordan 1961; Goldbach *et al.*, 2001), and also is an ultra-trace element for animals (Rowe and Eckhert 1999; Park *et al.*, 2004). It is revealed that boron takes part in structure of nucleic acid, carbohydrate and protein metabolism, sugar translocation, cell wall structure, membrane integrity and function, optimum growth and pollen germination

in plants (Iwai *et al.*, 2002; O'Neill *et al.*, 2004; Miwa *et al.*, 2007). Moreover, boron is also essential for vascular plants such as diatoms, marine algal flagellates, heterocystous cyanobacteria, actinomycetes of the genus *Frankia* and *Bacillus boroniphilus* which can tolerate more than 450 mM of boron (Ahmed *et al.*, 2007).

1. 5. What is the Importance of Boron?

1.5.1. The resistance and metabolism of boron in microorganisms and plants

For a long time, it is known that boron is required for plants to provide optimum growth. Boron level can be variously changed for different kinds of animals and eukaryotic organism that have also need boron to growth. For the rest, there is no information of boron-dependent organisms in litterateur so far except for cyanobacteria and *Bacillus boronophilus* (Ahmed, 2007). Boron has many active functions in bacteria, such as it is used to fix nitrogen by azotobacter, to be in signal molecules of quorum sensing and used to produce boron containing antibiotic called boromycin. Boron also had been used as a protector for nutrients since the 1950s. On the other hand, although it had been preferred as an antifungal agent in medicine, the mechanism of its toxicity could not be clarified; however it has been thought that boron binds to molecules that consist of cis-diol structure for example ATP, NADH and RNA, to put a stop to metabolic functions (Takano, 2007).

In the nature, boron concentration might reach to millimolar level especially near to the boron mine region and groundwater supplies. Therefore many kinds of organism, plant and yeast need to some mechanisms to survive under toxic level of boron concentrations (Takano, 2007).

Up to now, three pathway related to transportation of boron from membrane have been detected either in microorganism or plants. First of them is boron transportation from membrane with passive diffusion.

Second one is simplified boron transport with Major intrinsic protein (MIP) that has mission selective role to transport to water or not selective to carry for water and other neutral molecules. Finally, boron can be actively carried by transport proteins (Nozawa, 2006; Takano, 2007).

1.5.1.1 The resistance and metabolism of boron at microorganism

Boron effects on cyanobacteria were examined by a few research groups at different times. These studies show that boron is just effective on heterosite strain of cyanobacteria, and also required boron can be variable among each others (Bonilla, 1990). Eyster (1958) and Gerloff (1968) designed experiments on *Nostoc muscorum, Calothrix parietina, Anabaena cylindrical* and *Microcystis aeruginosa* to observe impact of boron during presence of dinitrogen and nitrate. The results showed that during presence of dinitrogen, boron stimulates growth of *N. muscorum, C. parietina* ve *A. cylindrical* that can fix nitrogen, and on the other hand boron cannot generate any differences on growth during presence of nitrate. Moreover, boron cannot effect at all on the growth of *M. aeruginosa* that does not have the ability to fix nitrogen (Ahmed, 2006; Warington, 1923)

Another study claimed that boron reacts with –OH groups that are internal layer of glycolipid in heterosite by cis-diols bonds to produce ester to provide stabilization. It was also predicted that boron deficiency causes various differences at envelope of heterosite, and that would be the reason of increasing O_2 diffusion to decrease nitrogenase activity (Garcia-Gonzalez, 1988).

Lewin revealed that *Cylindrotheca fusiformis* being sea diatoms needs boron for growth both at dark and bright medium. Furthermore, Lewin calculated that *C. fusiformis* must take minimumly 0.5 ppm boron to arrive maximum growth rate in first 10 h that correspond to a time of one generation. If boron concentration is under the 0.5 ppm level, the growth would be limited and this situation was observed first few days (Lewin, 1966).

1.5.1.2 The resistance and transport mechanism of boron in yeast

Living organisms are required to remove the boron from inside the cell by the mechanisms mentioned in the literature. Moreover, there are other unknown mechanisms which help to survive in environments containing toxic levels of boron. Living organisms show different resistance mechanisms to survive in environments containing boron. A lot of research is currently available in order to clarify the mechanisms of this resistance. Studies with yeast, *S. cerevisiae*, increased expression of *FPS1* and *BOR1* genes cause serious decrease in intracellular levels of boron, furthermore increased expression of *DUR3* gene was shown to be responsible of

boron acumulation intracellularly. Intracellular boron concentration is adjusted under the toxic-boron conditions by the presence of these three genes investigating to increase boron tolerance. Especially Bor1 protein encoded by *BOR1* gene actively remove excess boron from inside the cell. However, the physiological significance of these three genes is not clear at transport activity and the mechanisms that regulate the intracellular accumulation of boron is not known yet (Nozawa, 2006; Takano, 2007).

Recently, another study showed that the multidrug resistance *ATR1* gene encodes a protein with an important role as exporter of boron transport in the yeast.

A significant increase in gene expression levels of *ATR1* in toxic boron-containing environments provides resistance to boron in yeast cell by intracellularly reducing the level of boron (Kaya, 2009).

A study of *S. cerevisiae* mutants *hrb1*Δ and *rps20*Δ that do not exhibit high resistance level to boron, but mutant cells was determined to be more resistant compared to wild type. The inactivity of these genes was shown to increase the resistance of the boron in the *S. cerevisiae*. Moreover, proteins of Rps20 and Hrb1 are capable of attachment to RNA molecules. It is thought that the encoded proteins of these two genes are targeted by boron toxicity in yeast because of performing functions through binding sites of RNA. Boron and boron toxicity have not yet sufficient information and research on molecular mechanisms of resistance are not available. The identification of genes associated with resistance to boron will shed light on the molecular mechanism of it (Nozawa and Miwa, 2006).

It is also known that B is essential for plant development stage, if plants are affected by B deficiency manipulating enlargement and cell division in roots, they will also negatively be affected on flowering, fruit, and seed set among reproductive processes (Dell *et al.*, 1997; Tanaka *et al.*, 2008). *A. thaliana* needs boron for its developmental stage, so its roots consist of AtBOR1 protein involving in B xylem loading. During the absence of B; AtBOR1 is highly expressed; but when plant exposes to high B content AtBOR1 is degraded by post-transcriptional regulation (Takano *et al.*, 2005). Recently Ramon Perez-Castro and his co-workers found that VvBOR1 that is the grapevine B transporter and homolog to AtBOR1, and affects reproductive development of V*itis vinifera L.* VvBOR1 expression create

opportunity to accumulate B in grapevine berries, if there is any disruption on VvBOR1 in reproductive tissues, it will cause of occurrence of shot berries that is small and seedless. It was displayed that VvBOR1 is not completely same function like AtBOR1 with transformed to AtBOR1Δ yeast (Ramon, *et al.,* 2012). It can be suggested that VvBOR1 could play resembling role in reproductive tissues needing B to form flowers and berries in V*itis vinifera L.*; nearby a feasible molecular function in roots.

On the other hand; Boric Acid is also known with its antimicrobial activity making it a possible treatment agent for yeast vaginitis in alternative medicine (Prutting, *et al.,* 1998). The BA stops virulence effect by preventing transition *C.albicans* to hyphal growth that is pathogenic form (Moseley *et al.,* 2002). It has recently shown that overdose of BA damages cytoskeleton at the bud neck that cause irregular septa and bring with synthesis of aberrant cell wall protuberances. Cell increase the chitin synthesis against this matter to protect their homeostasis and repair cell wall (Schmidt *et al.,* 2002; Schmidt *et al.,* 2003).

The study of Martin Schmidt revealed that Slt2p playing a role in signal mechanism as a phosphorylation agent; is correspondingly increased during BA stress in yeast and *slt2*Δ mutant is highly sensitive to BA content (Schmidt *et al.,* 2010).

Moreover; another study aimed to identify boron transporter molecules in yeast. Unlike to boron efflux transporter AtBOR1 of *A. thaliana* and VvBOR1 of V*itis vinifera L.*; *S. cerevisiae* has also homolog ScBOR1 acting to export boron. Thus, *S. cerevisiae* strain lacking ScBOR1 has higher boron accumulation than wild type (Takano *et al.,* 2002).

The study of Nowaza and his co-workers aimed to understand exactly the role of Bor1 protein being export transporter for boron, and also clarified role of Dur3 and Fsp1 that were reported as a transporter of small molecules such as urea and glycerol. The boron accumulation in cell is higher than wild type for *bor1*Δ on the contrary of *dur3*Δ and *fsp1*Δ mutants. They have lower boron concentration inside the cell rather than wild type (Nozawa, *et al.,* 2006). It can be suggested that proteins encoded by *DUR3* and *FSP1* have role to uptake boron inside the cell, and work against to *BOR1* acting to export boron to provide homeostasis. However when *bor1*Δ, *dur3*Δ and *fsp1*Δ mutant were transformed with plasmids bearing to these genes, boron content

of cell is lower than the wild type for *BOR1* and *FSP1* deleted mutant. However, *dur3*Δ mutant accumulated higher amount of boron in their protoplasm than the wild type (Nozawa *et al.,* 2006).

It is again proposed that *bor1*Δ is sensitive to boron stress, but when *BOR1* is expressed by a multi-copy plasmid, they become tolerant against it.

On the other side, *dur3*Δ and *fsp1*Δ mutant can live easily under the toxic boron condition, but it is not valid for *DUR3* plasmid consisted mutant that cannot be alive during high level boron concentration.

1.6. Methods to Obtain the Desired Resistance of *S. cerevisiae* Against Different Stress Factors

The most important point in a study is determining the most accurate method to be used. A kind of inverse metabolic engineering method, evolutionary engineering methodology is an effective and successful method for the selection of individuals evolved as desired phenotypes with specific properties (Çakar, 2009).

1.6.1. Metabolic and inverse metabolic engineering

Metabolic engineering aims to develop cellular activities by enzymatic, transport and regulatory functions of the cell (Bailey, 1996). Metabolic engineering approach to obtain improved strains, transfer from outside the desired properties and metabolic pathway's genes that can give a new direction in the organism (Petri, 2004). With this method, it can be possible that any microorganisms produce the desired level of metabolites or acquired a desired property. To perform these procedures, many experimental approaches and many changes may be applicable to perform. Some of the gains are obtained by using metabolic engineering as follows;

- Improve production and / or efficiency
- Increasing intake of substrate
- Carbon sources that cannot be used as a substrate; can be used to make
- Improve the availability of an organism for fermentation processes
- Analysis and modification of metabolic pathways
- Elimination of unwanted or competitor pathways

- The development of the organism to resistance to toxic conditions (Strohl, 2001).

Metabolic engineering method is extremely successful in developing strains. However, this method has some drawbacks. In particular, at complex, unknown and not fully investigated cellular systems, it is extremely difficult to work with this method and generally is not possible. All of these challenges have led to the emergence of new approaches. One of the most important of one is inverse metabolic engineering method that consists of random and combinatorial approaches (Çakar *et al.*, 2005; Sonderegger, 2003).

Inverse metabolic engineering method is an alternative approach that eliminates the problems of metabolic engineering method (Bailey, 1996).

This method consists of three stages of the procedure. These stages are outlined as follows;

1. Obtaining of the desired phenotype
2. Determination of genetic or environmental factors for the desired phenotype
3. Creation or development of the desired phenotype at the organism or a different strain with directed genetic or environmental manipulations (Gill, 2003).

1.6.1.1. Evolutionary engineering strategy

It is based on the principle that cells of interest were first exposed to either chemical or physical mutagenesis. Then their desired phenotype was developed by selection of culture that contains a certain levels of stress. Unlike conventional selection methods; evolutionary engineering strategy based on using directed evolution rules to develop the microbiological properties (Çakar, 2009). It can be suggested that it is imitation of natural selection in evolution with repeated cycles of variation and selection of the desired phenotype stages. Variant cell populations are generated by directed evolution engineering method (Figure 1.8). Then the desired phenotype can be reached at the end of cycles of recurrent selection. In the next step for optimization studies the next target would be determined.

In general there are three specific stages in the strategy of evolutionary engineering. The first step of evolution engineering is selecting strains with desired properties.

In the second stage performance of this strain is analyzed at desired conditions. In the third stage, the next target is designed for optimizations in the future (Petri, 2004).

There are many examples of evolutionary engineering methods in the literature. The selection of mutant individuals' resistance to ethanol and acetate stresses and selected some of mutant individuals that can use xylose as a carbon source (Çakar, 2009 and Wisselink *et al.*, 2009).

1.7 Aim of the Study

In this study, physiological and molecular analysis of boron resistant *Saccharomyces cerevisiae* mutant that was previously obtained by evolutionary engineering method was aimed. In the evolutionary engineering step, the mutations had been created randomly by EMS agent and increasing boron content conditions were used to select the best resistant individuals. Decision of special individual mutant had been conferred with regard to boron stress resistance and cross-resistance to other stress types (Yilmaz, 2009). Furthermore, physiological analysis of the mutant was carried out by HPLC analyses, trehalose and glycogen determination including cell dry weight measurements. Glucose consumption and metabolite production rates were measured. The q-PCR analysis was performed to determine expression levels of some genes thought to be important for boron export mechanism with respect to previous studies in the literature. In these studies, by applying boric acid, effects of boron were observed. Besides, microarray analysis was performed to investigate whole genome expression levels of boron resistant mutant to study all mechanisms of boron resistance.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Yeast strains

The CEN.PK 113.7D (*MATa, MAL2*-gc, *SUC2*) and CEN.PK 113-1A (*MATα*) strains of *Saccharomyces cerevisiae* gently provided by Dr. Laurent Bendabis (INSA-Toulouse, Toulouse University, France) was used as a wild type. The wild type *S.cerevisiae* CEN.PK 113.7D was specially renamed as "905". Boron-resistant strain "BA8" which was obtained via evolutionary engineering by Dr. Ulkü Yılmaz (MOBGAM-Istanbul, Istanbul Technical University) was also used as mutant strain of *S.cerevisiae* CEN.PK 113.7D. Diploid cells (2n) were obtained by mating BA8 and opposite mating type wild type strain.

2.1.2 Yeast culture media and conservation conditions

In this study culturing processes were performed in two media. One of them was liquid media that culture was grown in 50 mL test tubes in 10 mL volume of YMM or YPD at 30ºC and 150 rpm. The other one was solid media in which cultures were grown on YMM or YPD plates including agar at 30ºC.

Stock culture was prepared by mixing the fresh culture with a 30% glycerol 'v/v'. Stock culture was prepared to save samples for long-term. Frozen cultures were prepared by adding 500 µl of culture to microfuge tubes and they were centrifuged at 10'000 *g* for 5 min. Then supernatant was removed and pellet was resuspended in 1 mL 30% (v/v) glycerol solution. If the culture was taken from a medium containing stress conditions, the culture was washed twice with YMM by following the same procedure. Finally, prepared cultures were kept at -80^{0}C deepfreeze for long-term storage.

2.1.2.1 Yeast minimal medium (YMM)

In this study, yeast minimal medium (YMM) was used for stress resistance determination, quantitative RT-PCR and transcriptomic analysis.

Table 2.1: YMM contents. *Agar is used for solid media.

Content "w/v"	Supplier	Amount
%2 D-Glucose	Fluka BioChemika	10 g
0.67% Yeast Nitrogen Base without aminoacids	Riedel-de Haen	20 g
%2 Agar*	Applichem	20 g
dH2O is added up to final volume 1000ml.		

2.1.2.2 Yeast extract peptone dextrose medium (YPD)

Yeast extract peptone dextrose medium is a complex medium used for regular growth of cultures.

Table 2.2: Ingredients of YPD. *Agar is used for solid media.

Chemical	Supplier	Amount
%1 Yeast Extract	Fluka BioChemika	10 g
%2 Dextrose	Riedel-de Haen	20 g
%1 Peptone	Riedel-de Haen	10 g
%2 Agar*	Applichem	20 g
dH2O is added up to final volume 1000ml.		

2.1.2.3 KAc (potassium acetate) medium

KAc medium includes only 1% 'w/v' anhydrous potassium actetate to force cell for sporulation by creating starvation condition. Strains undergo several divisions on sporulations medium and then sporulate after 3 to 5 days incubation at 30°C (Roth and Halvorson, 1969).

2.1.3 Chemicals

Chemicals used in this study were listed in Table 2.3.

Table 2.3: Chemicals used in this study.

Chemical	Company	Country
Nickel (II)-chloride-hexahydrate	Merck	Germany
Cobalt(II)-chloride hexahydrate	Merck	Germany
Sodium chloride	Riedel-de Haen	Germany
Ethanol (absolute)	J.T.Baker	Holland
Potassium acetate	Carlo Erba Reagents	Italy
Boron(II)Sulfate pentahydrate	Merck	Germany
Nickel(II)chloridehexahydrate(NiCl2.6H2O)	Merck	Germany
Glycerol	Carlo Erba Reagents	Italy

2.1.4 Laboratory equipment

Laboratory equipment used in this study are listed in Table 2.4.

Table 2.4: The general laboratory equipment used in this study.

Laboratory Equipment	Supplier
Micropipettes	Eppendorf (Germany)
Microcentrifuge	Eppendorf Microcentrifuge 5424 (Germany)
Benchtop Centrifuge	Beckman Coulter Allegra 25R Benchtop Centrifuge (USA)
Magnetic Stirrer	Labworld (Germany)

Table 2.4 (Continued) : The general laboratory equipments used in this study.

Laboratory Equipment	Supplier
Autoclaves	Tomy SX 700E (China)
Laminar Flow	Biolab Faster BH-EN 2003 (Italy)
UV-Visible Spectrophotometer	Shimadzu UV-1700 (Japan)
Light Microscope	Olympus CH30 (USA)
LightCycler 480 II	Roche (Switzerland)
Thermomixer Compact	Eppendorf (Germany)
Multiplate Spectrophotometer	BioRad Benchmark Plus (UK)
Desiccator	Finemech (USA) Bola-Star Vitrium Desiccator
Deep Freezes and Refrigerators	- 80˚C Heto Ultrafreeze 4410 (Denmark) - 20˚C Arçelik (Turkey) + 4˚C Arçelik (Turkey)
Ultrasonicator	Transsonic TP690
Vortex mixer	Heidolph (Germany)
Thermal Cycler	Techne TC-412 (UK)
Gel Electrophoresis	BioRad (UK)
Water Bath	Julabo SW22 (Germany)
Transilluminator	Vilber Lourmat
pH meter	Mettler Toledo MP220 (Switzerland)
Fluorometer	Invitrogen Qubit Fluorometer
HPLC System	
- System Controller	- Shimadzu SCL - 10A (Japan)
- Liquid Chromatography	- Shimadzu LC-10AD (Japan)
- Degasser	- Shimadzu DGU-14A (Japan)
- Refractive Index Detector	- Shimadzu RID-10A (Japan)
- Auto Injector	- Shimadzu SIL-10AD (Japan)
- Column Oven	- Shimadzu CTO-10AC (Japan)
- Column	- Aminex HPX-87H (300 x 7.8mm) Bio-Rad (USA)

2.1.5 Kits/enzymes, solutions/buffers, programs/ databases

Materials that were used for the experiments are listed according to their trademarks, and country of origin. Solutions, kits, buffers, enzymes, computer programs and databases are indicated in Tables 2.5-2.7, respectively.

Table 2.5: Kits and enzymes used in studies.

Kits	Supplier
DNA Isolation Kit	Roche High Pure PCR Template Preparation Kit (Switzerland)
RNA Isolation Kit	Qiagen RNA Purification Kit
cDNA Synthesis Kit	Roche Transcriptor First Strand cDNA Synthesis Kit (Switzerland)
SYBR Gold	Roche SYBR Gold Nucleic Acid Gel Stain (Switzerland)
Trehalase (from porcine kidney)	Sigma T8778-5UN (Germany)
A-Amyloglucosidase	Roche 3500U (Switzerland)
Glucose Oxidase/ peroxidase	Sigma (Germany)
o-Dianisidinedihydorochloride	Sigma (Germany)
Gluose Standard Solution	Sigma (Germany)
Amyloglycosidase (3500U)	Roche (Switzerland)
Master mix	GML Master Mix Kit
SYBR Green	Roche Light Cycler 480 SYBR Green I Master Mix (Switzerland)

Table 2.6: Solutions and buffers.

Solutions/Buffers	Contents/Amounts
TBE (electrophoresis buffer)	Tris (Hydroxymethyl) amino methane, boric acid, EDTA
PBS (Phosphate Buffer Saline)	pH 7.4 50mM
Agarose gel (%1.5)	1.5% agar in TBE
Glycerol (-80°CStrain Stock)	30%
Ethanol (Sterilization)	70%
HPLC Eluent (Mobile Phase)	0.5mM Sulphuric acid
Boric Acid Stock Solution	2M stock solution
SYBR Gold Solution	2µL SYBR Gold in 50ml TBE

Table 2.7: Programs/ databases and their functions.

Primer 3	Primer Design
Microsoft Office Programs	Data and result order
2100 Expert	Program of Bioanalyser for RIN measurement
www.yeastgenome.org	Genome and gene data base of *S. cerevisiae*
Gene Spring	Microarray data analysis

2.1.6 Primers for qRT-PCR and deletion experiments

Primers of the possible boron-related genes were designed by using Primer3-web v0.4.0 and Amplify3 v3.1 softwares. Sequences of the primers are shown in Table 2.8.

Table 2.8: Primers for q-PCR.

Primer name	**5' ➡ 3'**
ACT1 forward	CTTTCAACGTTCCAGCCTTC
ACT1 reverse	TCACCGGAATCCAAAACAAT
BOR1 forward	ACTTGGCTGGCATATGAACC
BOR1 Reverse	ATTCCTAGAACCCCGCTGAT
ATR1 forward	GTACCGAGGACCCAAAACAA
ATR1 reverse	ACCTATCACGCCCAAAACAG
DUR3 forward	TGCAGGAATGATGGTTTTGA
DUR3 reverse	TGCGAATGCAATAATTTGGA

2.1.7 HPLC stock solutions and HPLC standards

HPLC was used for quantitative determination of glucose, maltose, ethanol, glycerol and acetate. Standard solutions were prepared only for these metabolites. Glucose and each metabolite are listed in Table 2.10.Besides, 5mM sulphuric acid (H_2SO_4) was used as a mobile phase. Firstly, Solution A was prepared with glucose. Solution B was then prepared including ethanol, glycerol and acetate. Solution A and solution B were mixed at different rates. The last solution 'C', contained only maltose. Contents of stock solutions and standard solutions are shown in Table 2.10 and 2.11.

Table 2.9: Stock solutions of HPLC standards.

	Glucose			Add to dH2O up to Final volume 1000 mL
Solution A	120 g			
	Ethanol	**Glycerol**	**Acetate**	Add to dH2O up to Final volume 1000 mL
Solution B	30 g	2 g	4 g	
	Maltose			Add to dH2O up to Final volume 1000 mL
Solution C	4 g			

Table 2.10: Standard solutions of HPLC standards (Std: Standard solution).

Standards	Mixing Volumes	Eluent Volume	Final Volume
Standard 1	1 mL Solution A and 3 mL Solution B	2ml Eluent	6ml
Standard 2	0.75 mL Std1	0.25ml Eluent	1ml
Standard 3	0.50 mL Std1	0.50ml Eluent	1ml
Standard 4	0.25 mL Std1	0.75ml Eluent	1ml
Standard 5	0.125 mL Std1	0.875ml Eluent	1ml
Standard 6	0.063 mL Std1	0.937ml Eluent	1ml

Table 2.11: Standard solutions of HPLC standards.

Standard Solutions	Mixing Volumes	Eluent Volume	Final Volume
Standard 1	1ml Solution C	-	1 mL
Standard 2	0.75ml Std1	0.25 mL Eluent	1 mL
Standard 3	0.50ml Std1	0.50 mL Eluent	1 mL
Standard 4	0.25ml Std1	0.75 mL Eluent	1 mL
Standard 5	0.125ml Std1	0.875 mL Eluent	1 mL
Standard 6	0.063ml Std1	0.937 mL Eluent	1 mL

Table 2.12: Concentrations and retention time of metabolites in HPLC standards, retention times were given in ± 0.2 min range.

Metabolite(g/)	Glucose	Ethanol	Glycerol	Acetate	Maltose
Standard 1	20	15	1	2	20
Standard 2	15	11.25	0.75	1.5	15
Standard 3	10	7.5	0.5	1	10
Standard 4	5	3.75	0.25	0.5	5
Standard 5	2.5	1.875	0.125	0.25	2.5
Standard 6	1.125	0.9375	0.0625	0.125	1.25
RetentionTime(m)	8.43	19.95	12.5	13.81	6.95

2.2 Methods

2.2.1 Cultuvation conditions

Unless otherwise stated, yeast minimal medium (YMM) and yeast peptone-dextrose medium (YPD) were used to grow culture of yeast at 30°C, 150 rpm.

2.2.2 Diploidization of yeast

Diploidization process based on mating of *MATα* and *MATa* that was *S. cerevisiae* CEN.PK. In this study *S. cerevisiae* CEN.PK 113.1A *MATα* was used as opposite mating type wild type (named as 934 throughout in this study). First of all, 934 and BA8 strains were grown in YPD agar plate at 30°C overnight. The next day, both of strains were mixed equal volume on YPD solid medium. Zygotes were monitored under the light microscope by the end of 4-6 h of incubation at 30°C. In order to select single colonies, diploid cells were cultured to fresh YPD solid medium by streaking plate technique. Single colonies of diploid cells were cultivated on potassium acetate agar plate (anhydrous potassium acetate, 1% 'w/v') for 24-48 h at 30°C before the observation of tetrad formation under the light microscope.

2.2.3 Phenotypic characterization of wild type and boron-resistant mutant (BA8)

Phenotypic characterization was performed in order to indicate the cross resistance properties of boron-resistant *S. cerevisiae* mutant between boron and other metal and non-metal stresses. Precultures of BA8 and wild type (control group) were prepared to grown overnight at 30°C in 10 mL YMM. When the cells arrived to late log phase that is equal to 4 OD units, 1 mL volume of cells was collected from each culture by centrifugation and resuspend in 50 µL sterile distilled water. Serial dilution was done at 1:10 ratio for 4 times. Then 2.5 µL of each sample was spotted into solid YMM and YMM containing, 3 mM $CoCl_2$, 0.5 mM $NiCl_2$, 0.5 mM $CuCl_2$, 50 mM $B(OH)_3$, 80 mM $B(OH)_3$, 5% NaCl (w/v). They were incubated at 30ºC for 3 days. Furthermore, for the various metal stress experiments, spotting assay were also carried out for the following stress conditions; 3 mM $CrCl_3$, 20 mM $FeCl_3$, 0.5 mM $ZnCl_2$, 3 mN $CoCl_2$, 15 % NaCl.

2.2.4 Physiological characterization of wild type and boron-resistant mutant (BA8)

This experiment was planned to compare physiological characteristics of S.cerevisiae wild type and boron-resistant mutant 'BA8' in the absence and presence of B(OH)3 concentrations.The first step was designed to analyze growth curve for wild type and mutant called BA8. A preliminary experiment was performed to estimate which boron concentration would be applied to both wild type and BA8 cultures. 90 mM B(OH)3 was chosen for initial boron concentration to start growth curve analysis. Growth curve of wild type and mutant cultures both in the presence and the absence of boron were realized. One day before preculture of wild type and mutants were prepared from the stock culture to grow overnight at 30°C and150 rpm. Then both of cells were inoculated into YMM with an initial OD600 of 0.25. Incubation process was followed in 4 flasks containing 400 mL medium at 30°C and 150 rpm for 48h. Two flasks just consisted ofYMM and the others included YMM that contained 90 mM B(OH)3. Cell growth was monitored by measuring OD600 values and growth curves of the cultures were plotted based on the OD600 values versus their corresponding sampling time as hour. Supernatant and pellet samples were also taken to use for next step experiments which consist of extracellular metabolites analysis by HPLC analysis, CDW and Trehalose-Glycogen content determination (Figure 2.1). Supernatant samples would be used for HPLC analysis on the other hand; pellet samples would be used to calculate CDW (mg/mL) values.

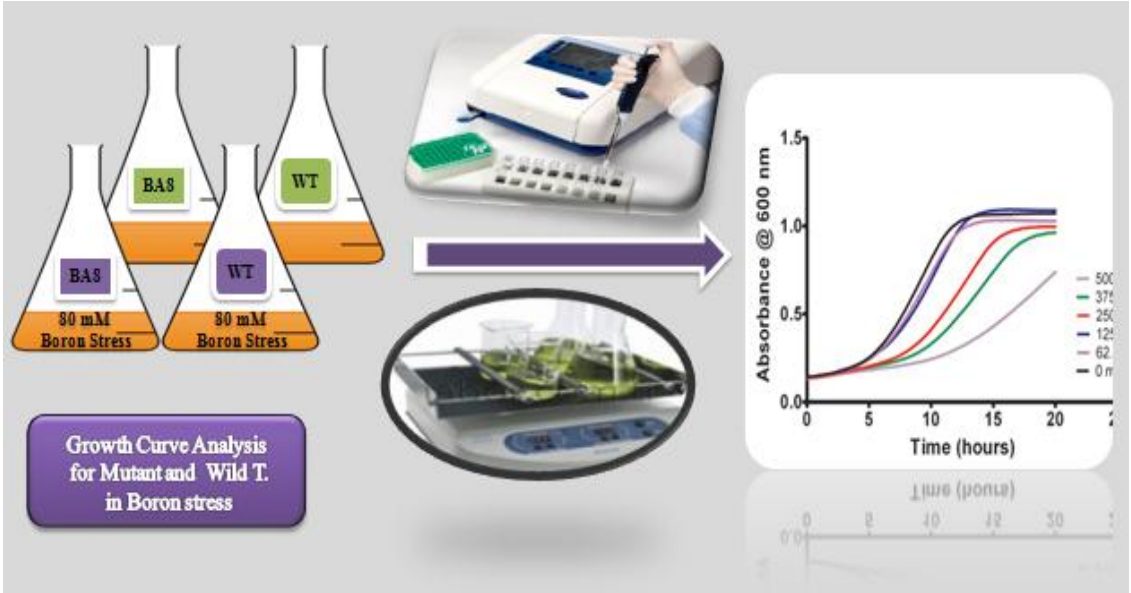

Figure 2.1: Schematic representation of Growth curve, HPLC and CDW analysis.

2.2.4.1 Analyses of metabolite profiles by HPLC during batch cultivation

Microorganisms have convertion or production of extracellular metabolites during their cultivation proceses that could be detected and analyzed by HPLC (high-performance liquid chromatography) cultivation. HPLC was used to determine ethanol, acetate and glycerol production and glucose consumption profile of samples in absence and presence of $B(OH)_3$. During the batch cultivation, 1.5 mL of samples was also taken for HPLC analysis. Each sample was centrifuged for 5 min at 10'000 *g*. Supernatant was filtered through a 0.2 µm filter and kept at -20°C. For HPLC analysis, 500 µl of samples and 6 known standard solutions were transferred into vials. HPLC analysis of metabolites was carried out using an Aminex© HPX-87H column at 60˚C with a flow rate of 0.6 mL/min and using a Shimadzu RID-10A refractive-index detector with 20 µL injections from samples. 5 mM sulphuric acid solution was used as mobile phase.

HPLC analysis consisted of manual calculation to determine the concentrations of each metabolite of samples with drawing calibration graph of standard solutions giving a peak area versus concentration. The interpolation method was used to calculate the unknowns' concentration of each metabolite.

2.2.4.2 Determination of trehalose and glycogen content

The aim of this process was to detect quantative amounts of glycogen and trehalose via enzymatic assay (Parrou and François, 1997) on the samples which were collected on the batch growth of the cultures as explained in Section 2.2.5. The process was performed by harvesting 25 OD unit of cells at selected time points during the batch cultivation. The supernatants were removed and pellets were resuspended in 250 µL 0.25 M Na2CO3 in screw-top microcentrifuge tubes. Then tubes were incubated at 95°C for 4 h. 1 M 150 µL acetic acid and 0.6 mL 0.2 M Na-acetate (pH 5.2) was added onto the cell suspension. Half of this suspension (500 µL) was transferred to new tubes and incubated overnight with 10 µL trehalase at 37°C. On the other hand, second half of the suspension was incubated overnight with 20 µL alpha-glycosidase at 57°C in a rotary shaker. One day later both of incubation suspensions were centrifuged at 5000 g for 3 min. 96-well plate was used to mix 20 µL of each sample and five glucose standard solutions with 200 µL glucose oxidase solution. Incubation contiuned upon 30 min at 37°C and dark. Microplate Reader

was used to read the absorbance of sample at 490 nm. In order to determine the glucose content of samples, absorbance of standard solutions was recorded and a calibration graph of absorbance versus concentration was drawn. The amount of unknowns was then found using the interpolation method.

2.2.4.3 Cell dry weight (CDW) measurement

Two days before batch cultivation sufficient microfuge tubes were labelled with number and incubated at 80ºC. Then each tube was weighed to determine tare of empty tubes. During batch growth of the cultures, 2 mL sample was taken and centrifuged at 14'000 *g* for 5 min to thrown away supernatant. Subsequently microfuge tubes containing the pellets were dried at 80°C for 48 h and placed in a desiccator for 30 min. After all dried tubes were reweighted with dry pellet to compare with first weight. The difference of the empty and pellet-containing tubes were accepted as CDW as mg.

2.2.5 Molecular characterization of boron-resistant mutant BA8

2.2.5.1 Culturing cells for genetic analysis

First of all preculture was prepared in 20 mL YMM in 100-mL flask and they were incubated at 30°C and 150 rpm to perform microarray analysis for the wild type and boron-resistant mutant 'BA8'. After the overnight incubation, they were inoculated into 100 mL fresh YMM in 500-mL flask with the initial OD_{600} of ~0.1 and they were incubated at 30°C and 150 rpm. RNA isolation was performed when cultures reaches an OD_{600} of about 1 ($5x10^7$ cells/mL). RNA isolation was performed via Qiagen High Pure RNA Isolation Kit according to the manual procedure.

2.2.5.2 Quality and quantity of isolated RNA

UV-Vis Spectrophotometer (NanoDrop 2000, USA) and Agilent 2100 BioAnalyser were used as a measuring device to detect RNA content 'ng/µL' of the samples and RNA integrity 'RIN'.

Purified RNA was measured by Nanodrop 2000 (ThermoScientific) and analyzed by BioAnalyzer 2100 (Agilent technologies). Then Agilent 6000 Nano Kit was used by following the instructor's manual in order to determine RIN of RNA samples.

2.2.5.3 Microarray analysis

The scheme of microarray analysis procedure is displayed in Figure 2.2. The experiment inculed 3 crucial steps. First step was cRNA synthesis and labelling the nucleic acids with single colour dye (-Cy3). Second step was hybridization of the samples to Agilent Microarrays. After the hybridization step, slides were cleaned from unconnected samples to scan microarray. DNA Microarray Scanner (Agilent Technologies) was used to detect signals of microarray slides. At the end, Genespring program was selected to evaluate data.

All the genes whose expression changed more than two-fold were classified into clusters by using FunSpec and Funcat online softwares [URL-1 and 2].

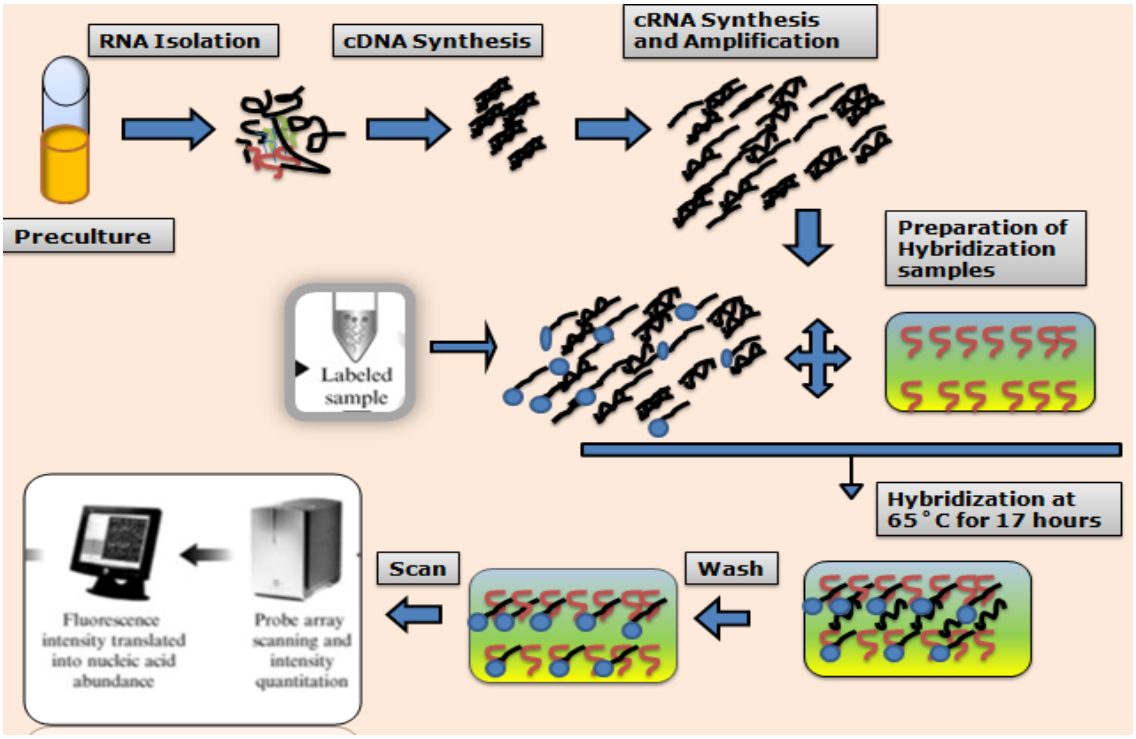

Figure 2.2: Microarray procedure.

2.2.6 Determination of expression patterns of boron-related genes

Quantitative Real-Time PCR (qRT-PCR) was performed to compare expression level differences between boron-resistant mutant "BA8" and wild type in terms of boron-related genes, mentioned in the literature.

2.2.6.1 RNA isolation for qRT-PCR

RNA isolation was performed in absence and presence of the boron for boron-resistant mutant "BA8" and Wild type. Firstly, precultures were prepared into 20 mL YMM in 100 mL flasks and they were incubated at 30°C and 150 rpm. Next day, all samples culture media were inoculated to 100 mL fresh YMM in a 500 mL flask to an initial OD_{600} of 0.2. They were placed to incubate at 30°C and 150 rpm. All samples were grown until their OD_{600} value was around 3. After that 1 OD unity of cells were collected to 1.5 mL volume microfuge tubes. Following that the extraction was applied according to the RNeasy Mini Kit (QIAGEN) procedure. Isolated RNA content 'ng/µL' of the samples was determined by using Micro-Volume UV-Vis Spectrophotometer (NanoDrop 2000, USA). Finally RNA samples were stored at -80°C.

2.2.6.2 cDNA synthesis

In order to perform qRT-PCR analysis to determine expression level of target genes, cDNA synthesis is crucial because mRNAs are target to detect expression pattern. Because of that, mRNA content was converted to cDNA. First of all, the initial RNA amount was set to 1000 ng for each sample before starting to cDNA synthesis. In the next step manual procedure of Transcriptor First Strand cDNA Synthesis kit (Roche) was followed to perform 2 stage of process. At the begining, the volume of each sample was calculated so as to 1000 ng. 1 µl anchored-oligo (dT)18 primer and water were added to complete volume to 13 µL. The suspensions were then incubated at 35°C for 10 min to provide binding condition for primer. In the second stage 7 µL reverse transcriptase mix was added to each sample tube. The mixture of each sample with a final volume of 20 µL was incubated at 55°C for 30 min and then at 85°C for 5 min to inactivate the Transcriptor High Fidelity Reverse Transcriptase. Synthesized RNA samples were stored at -20°C. cDNA content 'ng/µL' of the samples and cDNA integrity were determined by using Micro-Volume UV-Vis Spectrophotometer (NanoDrop 2000, USA)

2.2.6.3 QRT-PCR (Real Time) analysis

All process of expression level determination is depicted on Figure 2.3. Green chemistry (Light Cycler 480 SYBR Green 1 Master Kit, Roche, Cat no: 04 707 516

001) was used to amplify products of the potential boron-related genes in Light Cycler 480 Equipment (Roche). The 20 µL reactions were performed to analyze expression patterns (Table 2.13). During this process *ACT1* (β-actin) was selected as the reference gene to compare expression levels. Cycling conditions were listed in Table 2.14. On the otherhand comparative C_T method [$2^{-\Delta\Delta CT}$] was used to calculate qPCR data analysis. This method has been investigated relative gene expression of a target gene with respect to an internal control gene'expression level (Schmittgen and Livak, 2008).

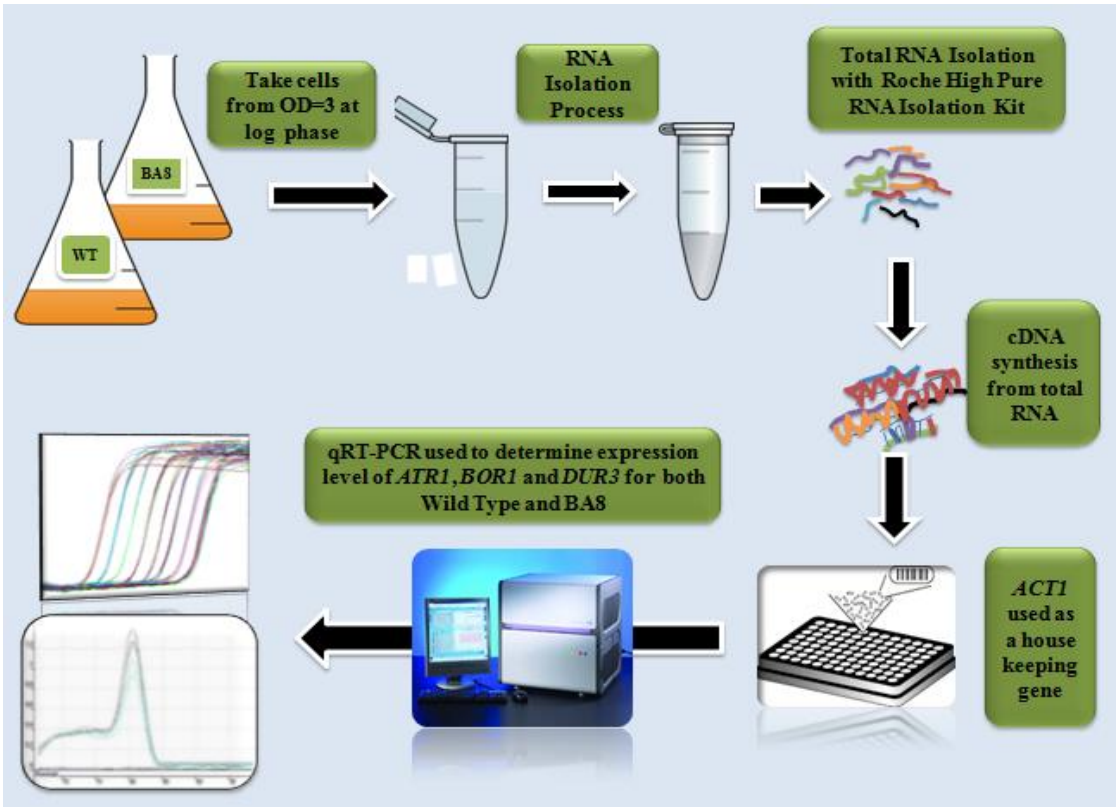

Figure 2.3: Total RNA Isolation, cDNA shynthesis and QRT- PCR for ATR, BOR1and DUR3 genes.

Table 2 13: Component of Real Time PCR mixture.

Component	Amount	Concentration
Primer (Forward)	1µL	10 µM
Primer (Reverse)	1µL	10 µM
cDNA	8µL	
SYBR Green	10µL	

Table 2.14: Cycling conditions for qPCR program.

Stages		**Temperature (**°C)	**Time**	**Cycle Number**
Initial Denaturation		95	10 min	1
Amplification =	Denaturation	95	10 sec	45
	Annealing	54	15 sec	
	Extension	72	20 sec	
Final Extension		95	1 min	1
Melting		65	1 min	
Cooling		40	10 sec	1

3. RESULTS

3.1 Cross-Resistance Analysis of Boron-Resistant Mutant

Cross-resistance in boron-resistance mutant was carried out by spot assay to find any possible relationship between boron and other metal or non-metal stresses.

3.1.1 Phenotypic analysis by spot assay

In order to determine qualitatively the cross-resistance level of boron-resistance spot assay was performed. During this process cultures were exposed to several stress conditions such as 3 mM $CoCl_2$, 0.5 mM $NiCl_2$, 0.5 mM $CuSO_4$, 50mM $B(OH)_3$, 80mM $B(OH)_3$ and 5% NaCl. Image of the solid plates are displayed in Figure 3.1, 3.2, 3.3 and 3.4 upon 48 h incubation at 30°C. Each row was diluted from 10^{-1} to 10^{-4}-fold that was shown from left to right in each plate.

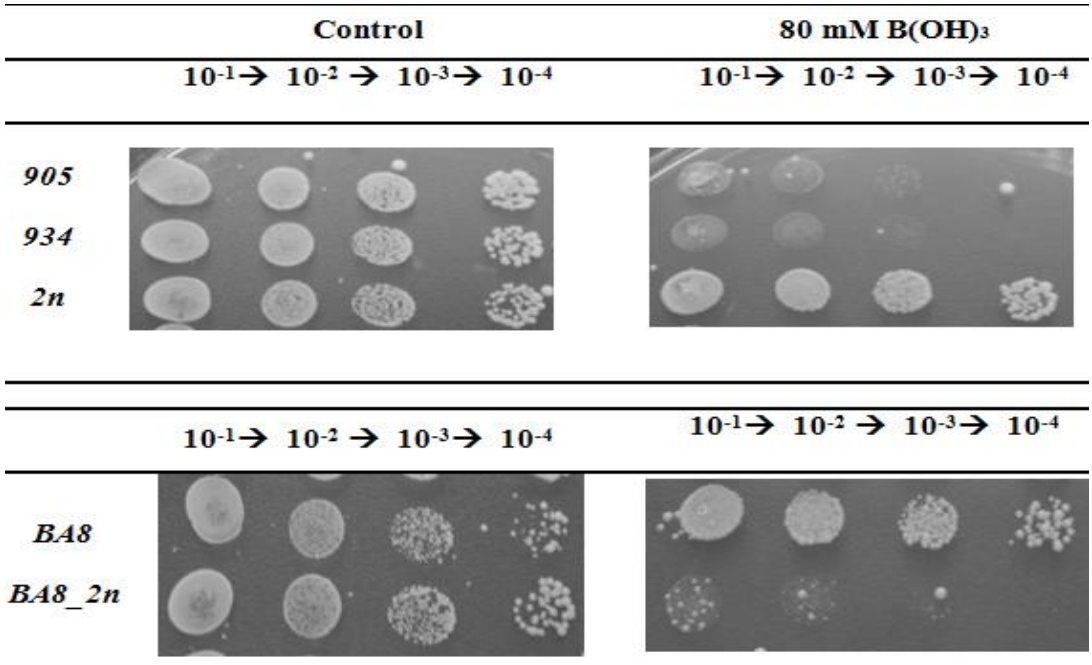

Figure 3.1: Wild types with opposite mating types (905 'MATa' and 934 'MATα'), diploid obtained from the genetic cross of 905x934, Boron-resistant mutant "BA8" and diploid obtained from the genetic cross of wild type. Cultures were diluted from 10-1 to 10-4-fold and incubated in control and 50 mM B(OH)3- 80 mM B(OH)3 containing medium.

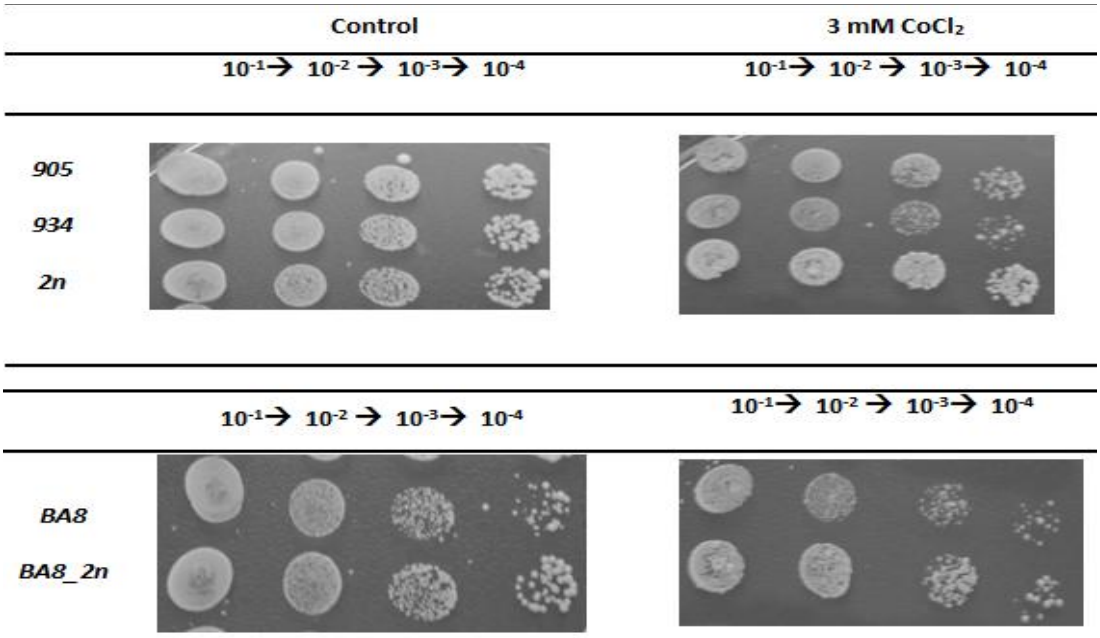

Figure 3.2: Wild types with opposite mating types (905 'MATa' and 934 'MATα'), diploid obtained from the genetic cross of 905x934, Boron-resistant mutant "BA8" and diploid obtained from the genetic cross of wild type and BA8 were diluted from 10-1 to 10-4-fold and incubated in control and 3 mM CoCI2 containing medium.

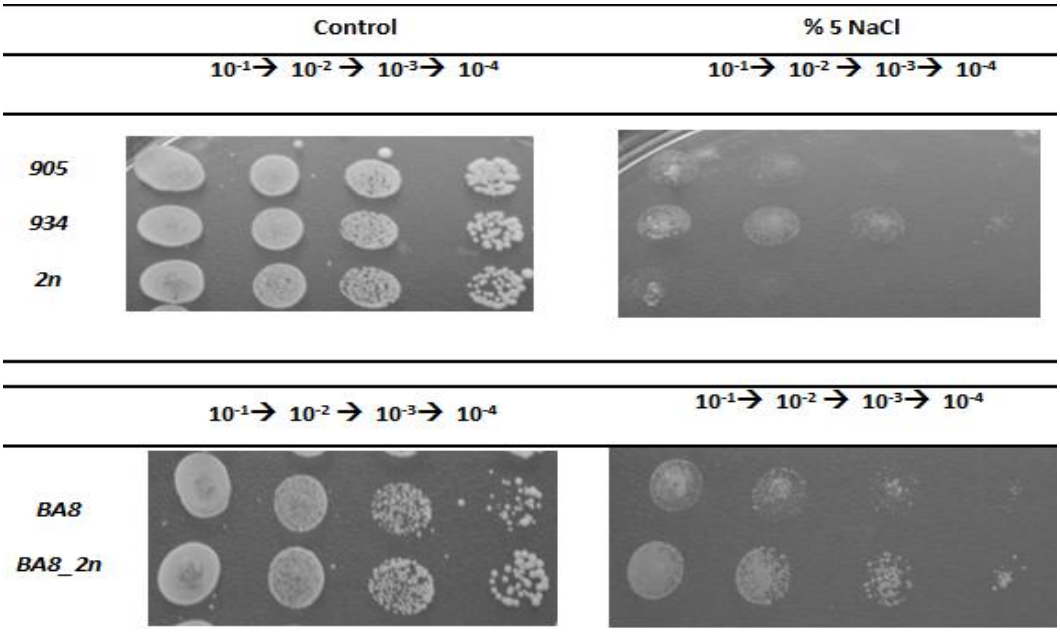

Figure 3.3: Wild types with opposite mating types (905 'MATa' and 934 'MATα'), diploid obtained from the genetic cross of 905x934, Boron-resistant mutant "BA8" and diploid obtained from the genetic cross of wild type and BA8 were diluted from 10-1 to 10-4-fold and incubated in control and %5 NaCI.

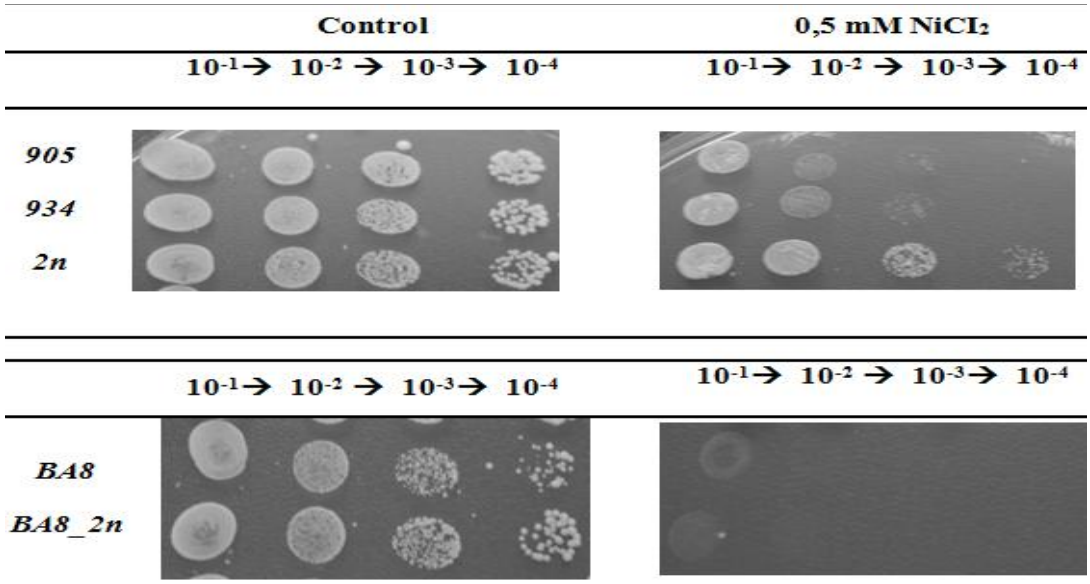

Figure 3.4: Wild types with opposite mating types (905 'MATa' and 934 'MATα'), diploid obtained from the genetic cross of 905x934, Boron-resistant mutant "BA8" and diploid obtained from the genetic cross of wild type and BA8 were diluted from 10-1 to 10-4-fold and incubated in control and 5 mM NiCI2.

The results of spot assay were revealed that BA8 and 2n (934xBA8) gained cross-resistance to cobalt and NaCl. Otherwise they could not tolerate nickel treatment. Although the diploid 2n (905x934) obtained from the wild types with opposite mating types grown normally in the presence of boron, 2n (934xBA8) could not survive upon boron treatment (Figure 3.5). On the other hand; 905, 934 and especially 2n (905x934) had the capability of tolerating cobalt and nickel stress applications (Figure 3.5).

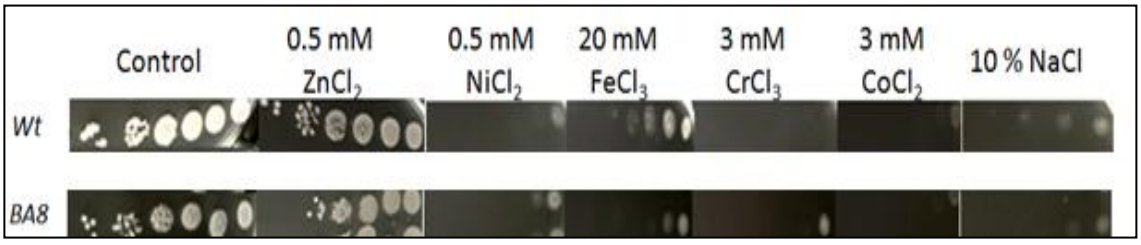

Figure 3.5: Cross-resistance results of boron resistant S. cerevisiae mutant individual BA8 and wild-type. Cell cultures were grown at 30°C for 3 day on solid YMM plates with and without stress conditions, after serially diluted from 100 to 105 (from right to left). Possible resistance to $ZnCl_2$, $NiCl_2$, $FeCl_3$, $CrCl_3$ and $CoCl_2$ metal and salt (NaCl) osmotic stress factors were investigated.

3.2 Physiological Characterization of Wild Type and Boron-Resistant Mutant

3.2.1 Batch cultivation of wild type and BA8 mutant

Boron-resistant mutant 'BA8' and wild type were examined for their physiological traits by cultivating them in aerobic-batch condition in the presence and absence of boron during 56h. The growth curve analysis (Figure 3.6) was performed by taking OD600 samples every one and half hour as triplicates. Moreover the maximum specific growth rates (Table 3.1) were calculated from the $lnOD_{600}$ data of the samples (Figure 3.7)

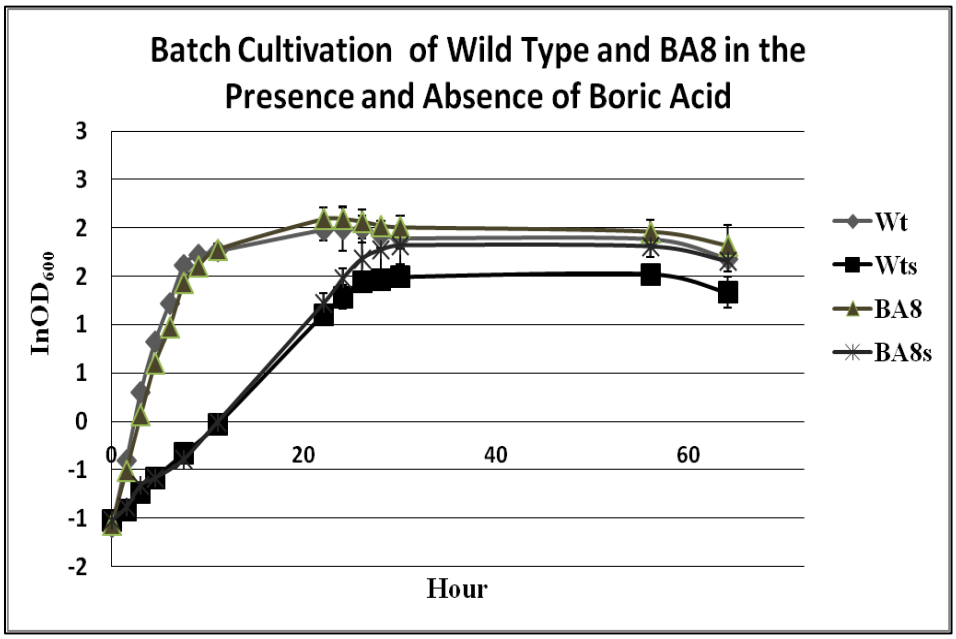

Figure 3.6: Wild type and BA8 were cultivated in the absence and presence of 80 mM B(OH)3 at 30ºC and 150 rpm.

Table 3.1: Maximum specific growth rate of Wt and BA8 in the presence and absence of boron.

Organism	**Growth rate (μ, h^{-1})**	
Wt	0.43	**Wts/Wt**
BA8	0.37	0.21
Wts	0.09	**BA8s/BA8**
BA8s	0.11	0.30

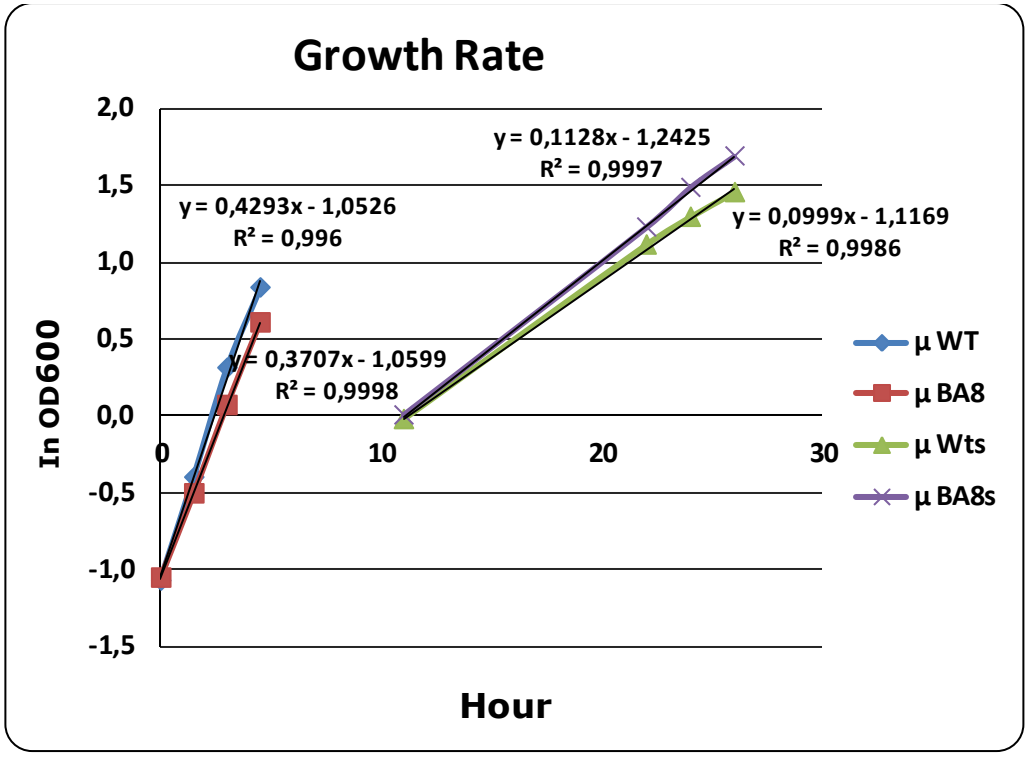

Figure 3 7: Maximum specific growth rate (h-1) of both BA8 and wild type was calculated for aerobic-batch condition in the presence and absence of boron.

3.2.2 Metabolite consumption and production profile during batch cultivation

HPLC was performed to detect changes in the concentration of ethanol, glycerol, maltose and acetate which are important metabolites of yeast strains. Moreoever, glucose consumption was monitored through batch cultivation.

The aim of this process was to detect differences of metabolite profiles in the presence and absence of boron stress both for BA8 and wild type. The standard curves were obtained for glucose, acetate, ethanol, maltose and glycerol. The equations obtained from these curves were used to calculate corresponding metabolite content of the samples (g/L). The glucose consumption and glycerol, acetate, maltose and ethanol production profile of wild type *S.cerevisiae* and BA8 were determined as a function of time and the corresponding graphs are shown in Figure 3.8 respectively.

The growth, glucose consumption and glycerol, acetate and ethanol production were represented in the same graph for wild type and BA8 in the presence and absence of boron to reveal the relationship between each other's (Figure 3.9 and 3.10).

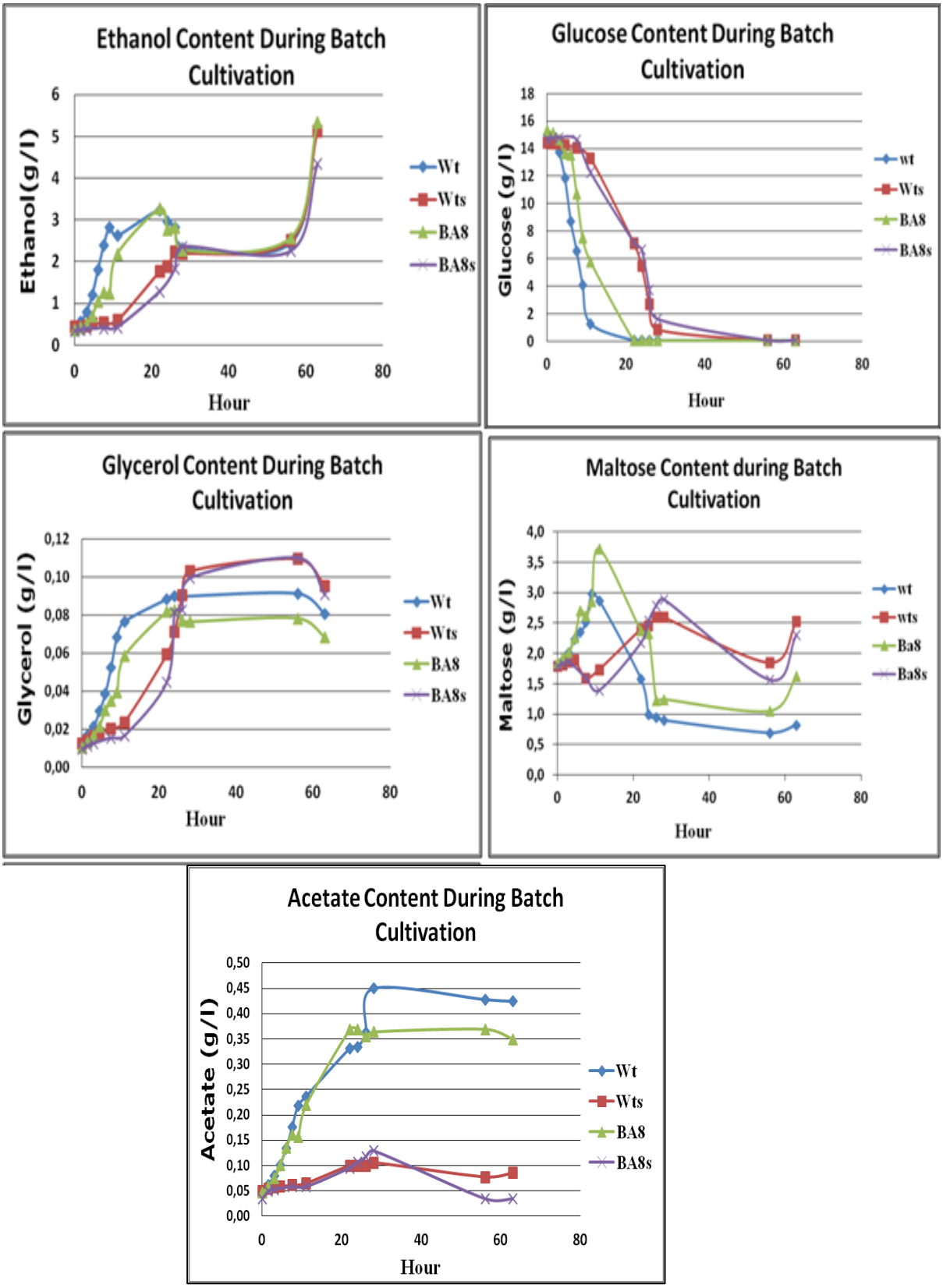

Figure 3.8: Glucose utilization and glycerol, acetate, maltose and ethanol production profiles of BA8 and the wild type were determined for the boric acid-treated (Wts and BA8s) and non-treated (Wt and BA8) samples.

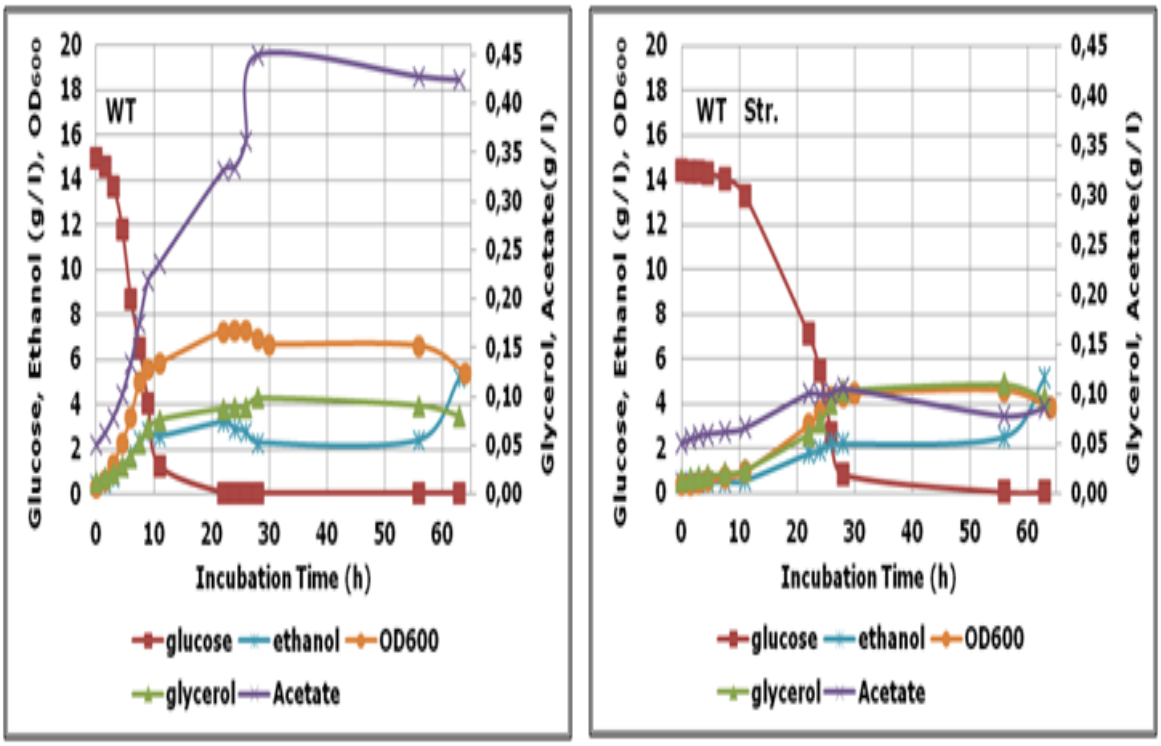

Figure 3.9: Extracellular metabolite concentrations' changes and growth of wild type upon time in the presence and absence of boron stress. WT stands for wild type and WT Str. indicates the wild type samples treated with boron.

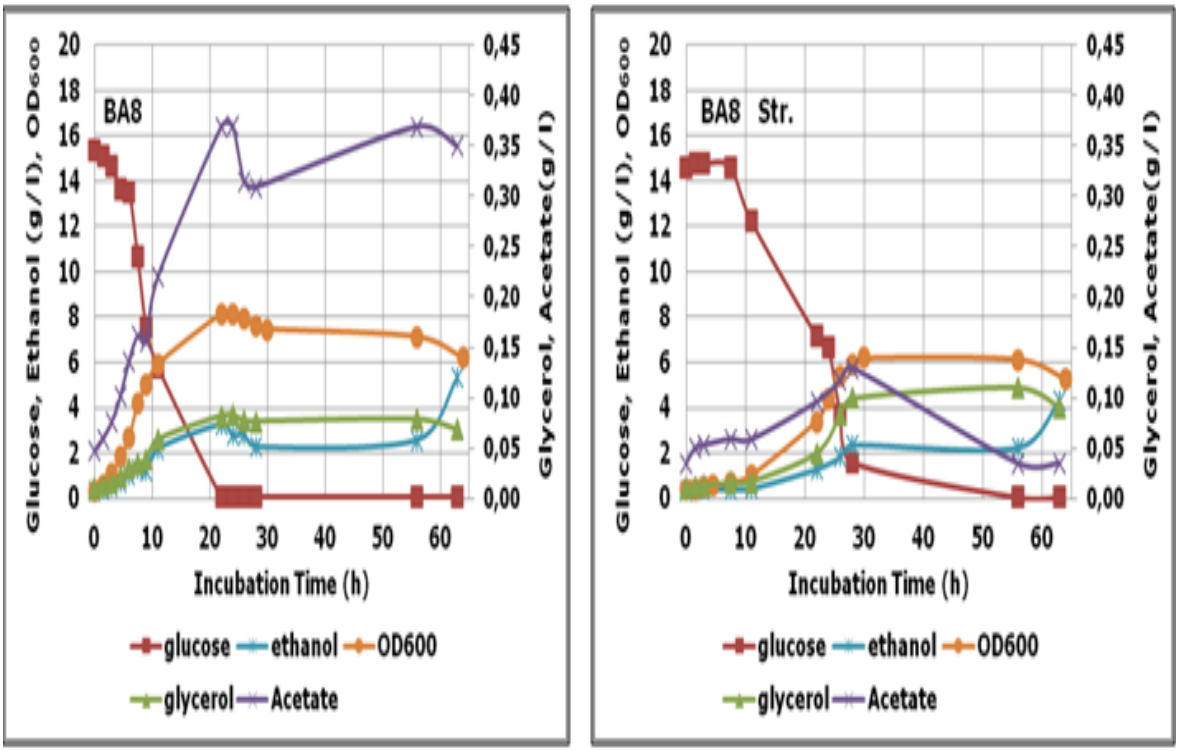

Figure 3.10: Extracellular metabolite concentrations and growth of BA8 mutant during batch growth conditions in the presence and absence of boron stress.

3.2.3 Cell dry weight (CDW) analysis for wild type and BA8

Cell dry weight was determined from the pellet samples that were taken during growth curve analysis. The result of cell dry weight was calculated by subtracting tare of empty tubes from that of the dried cell-containing weights. The results of CDW/mL were displayed in Figure 3.11 and 3.12, respectively.

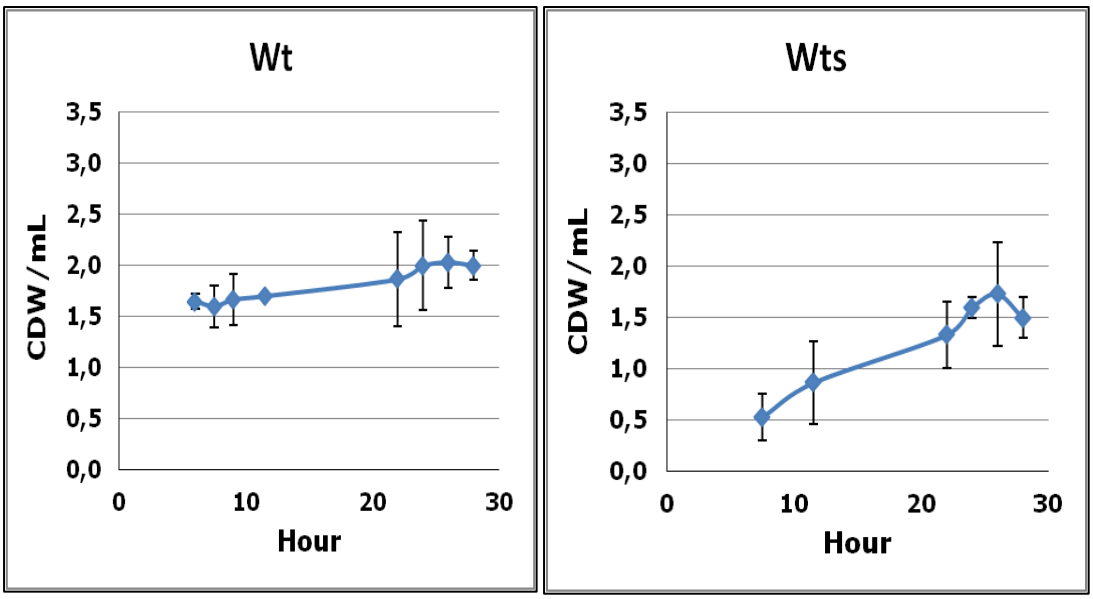

Figure 3.11: Cell dry weight of Wild type during batch cultivation in the presence and absence of boron.

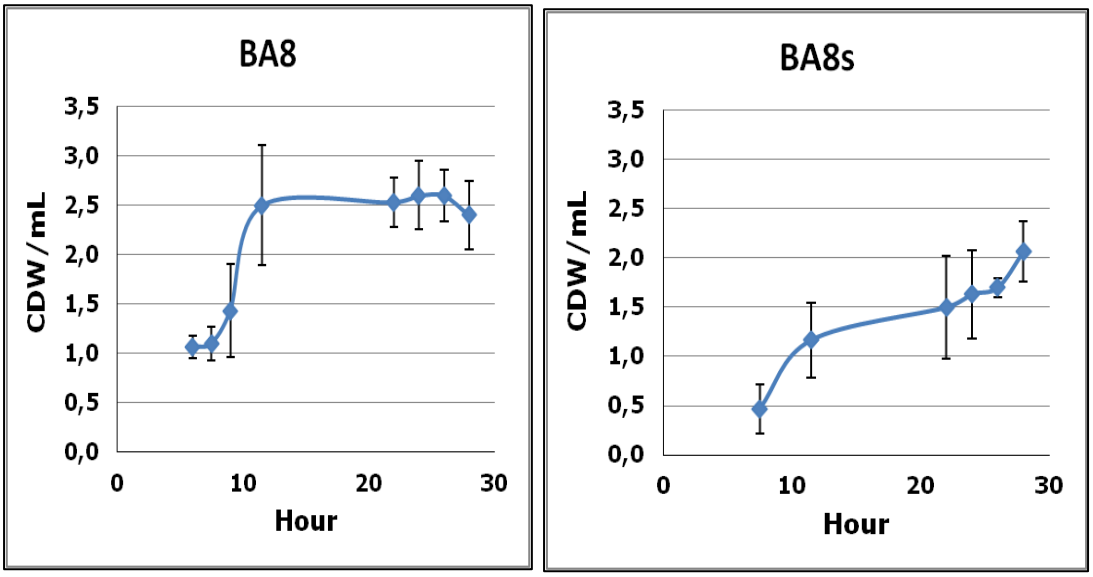

Figure 3.12: Cell dry weight of BA8 mutant during batch cultivation in the presence and absence of boron.

3.2.4 Determination of trehalose and glycogen content

The enzymatic assay as explained in Section 2.2.5.2 was performed to determine the content of intracellular reserved carbohydrates (trehalose and glycogen) of wild type and BA8 in the presence and absence of boron stress. Samples were taken at irregular time intervals by monitoring the specific growth of each strain. Results are given in Table 3.2 and 3.3 and are graphed in Figure 3.13 and 3.14.

Table 3.2: Trehalose production for BA8 and Wt in the absence and presence of boron condition

Trehalose Hour	wt	Hour	wt stress	Hour	BA8	Hour	BA8 stress
6	0.02	9	0.02	6	0.15	9	0.07
9	0.05	21	0.22	9	0.43	21	0.14
21	0.06	24	0.2	21	0.73	24	0.72
24	0.03	27	0.15	24	0.85	27	0.78
30	0.02	30	0.21	30	0.88	30	0.67

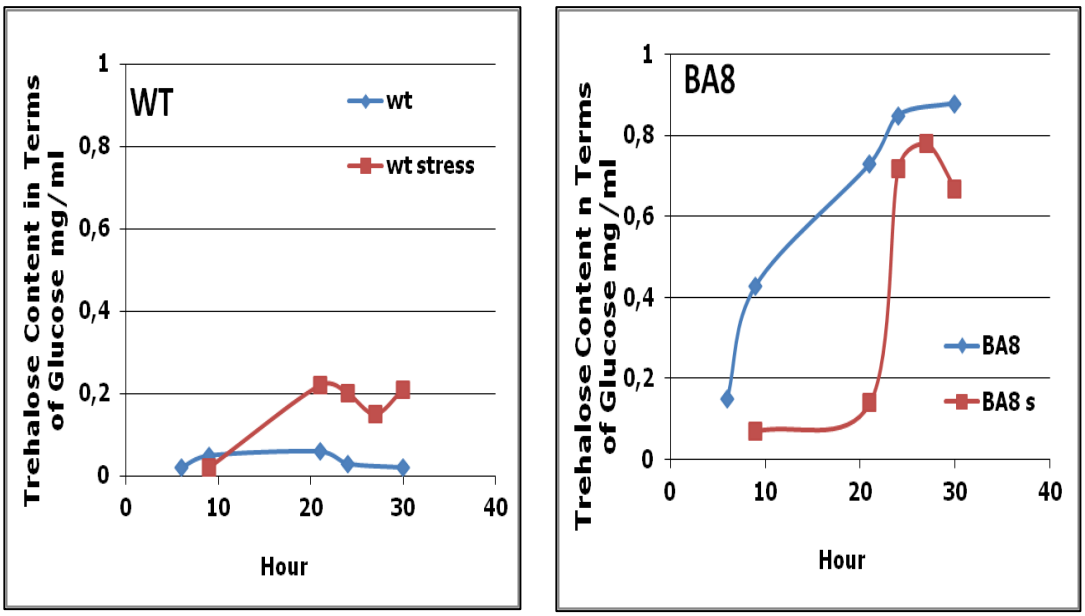

Figure 3.13: Differences of trehalose production for BA8 and Wt absence and presence of boron stres

Table 3.3: Glycogen production for BA8 and Wt in the absence and presence of boron condition

Glycogen Hour	wt	Hour	wt stress	Hour	BA8	Hour	BA8 stress
6	0.07	9	0.18	6	0.11	9	0.11
9	0.03	21	0.04	9	0.19	21	0.17
21	0.05	24	0.09	21	0.2	24	0.31
24	0.05	27	0.04	24	0.34	27	0.26
30	0.04	30	0.04	30	0.25	30	0.16

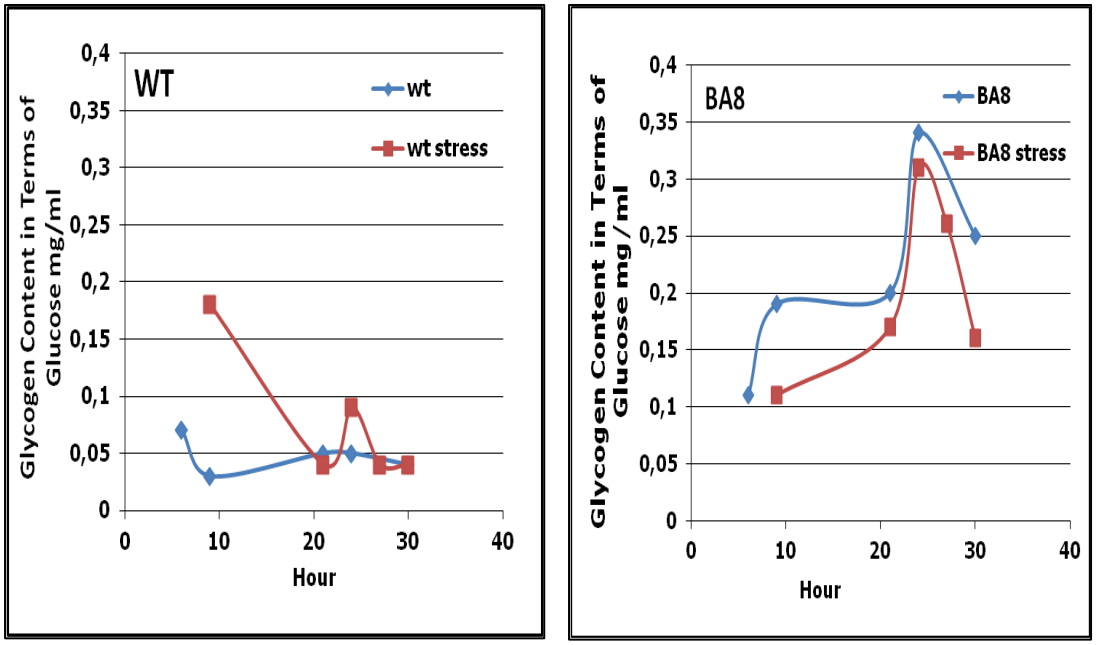

Figure 3.14: Differences of glycogen production for BA8 and Wt absence and presence of boron stress.

3.3 Expression Level Determination of Wild Type *Saccharomyces cerevisiae* and Boron-Resistant Mutant (BA8) Strains by qRT-PCR

Quantitative Real-Time PCR were realized for some genes implicated in boron resistance for both BA8 and wild type under stress-treated and control conditions. Before cDNA synthesis, total RNA amounts, which were isolated from wild type and

mutant BA8, were measured by Nano Drop instrument (Table 3.4). Reliability of the experiments was assessed in an experiment with dilution series of cDNA template (10^{-1} to 10^{-3}) to create a standard curve of the change in Ct value vs. Standard curves were required to calculate efficiency, slope and error values of experiment by software program as mentioned in Section 2.2.7.3 (data not shown). According to Dorak (2006) the efficiency and slope values of Standard curves must be in the range of 1.9-2.1 and -3.1,-3.6, furthermore error values also must be below 0.2. Calculated expression levels were normalized to the expression level of untreated wild type cells.

Table 3 4: Measurements of RNA concentration as isolated from BA8 and Wild type at OD600=3 in the presence of boron condition by Nano Drop instrument

Strain	RNA Conc. (ng/µL) OD_{600} = 3
Wild Type Control	**507.1**
BA8 Control	**131.6**
Wild Type Stress	**96.2**
BA8 Stress	**277.6**

3.3.1 qRT-PCR analysis for boron homeostasis genes

Total RNA from wt and BA8 mutant of *S. cerevisiae* was performed by method as mentioned in Section 2.2.7.1 for determining and comparing the expression levels of *BOR1, ATR1* and *DUR3* genes (Kanazawa *et al.,* 1988 and Jennings *et al.,* 2007) which are linked to boron transport.

BOR1 encodes localized plasma membrane protein; Bor1p that exports excess boron out of the cell to maintain ionic homeostasis inside the cell (Sá-Correia et al., 2008). *BOR1* gene expression levels of strains are shown in Figure 3.15.

BOR1 was downregulated in boron treated wild type and BA8 cells whereas it was upregulated in absence of boron at BA8.

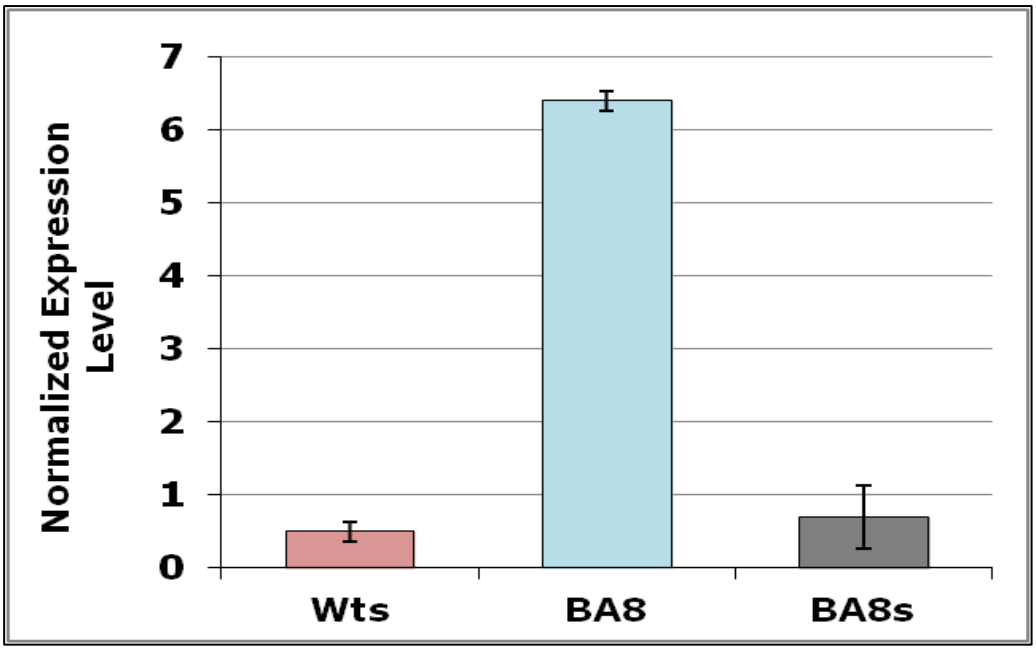

Figure 3.15: Normalized 2-ΔΔCT as fold expression level of BOR1 gene.

ATR1 which was identified later plays a major role to export boron out of the cell, when it is upregulated by high levels of boron (Kaya *et al.*, 2009). *ATR1* gene expression levels of strains are shown in Figure 3.16.

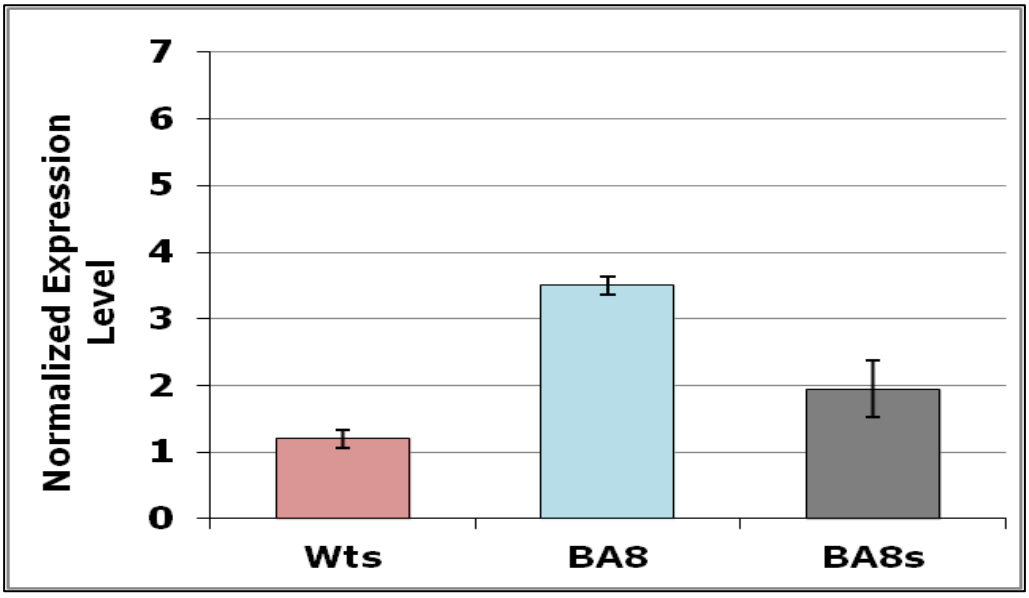

Figure 3.16: Normalized 2-ΔΔCT as fold expression level of ATR1 gene.

Expression level of *ATR1* in the boron treated wild type and BA8 cells were increased. However, BA8 expresssed *ATR1* gene in absence of boron condition higher than boron untreated cells.

On the contrary, Dur3 transporter protein has a role against the efflux transporters Bor1 and Atr1 (Jennings *et al.*, 2007 and Takano *et al.*, 2005). Although its functions are not clear, overexpression of *DUR3* increased boron levels inside the cells (Nozawa *et al.*, 2006). *DUR3* gene expression levels of strains are shown in Figure 3.17.

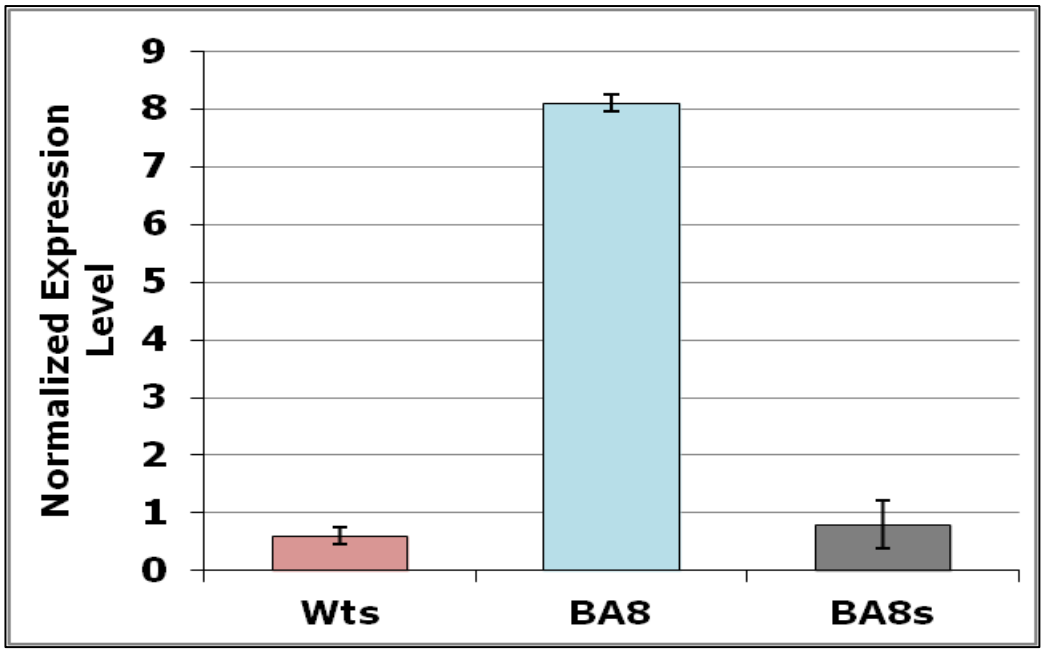

Figure 3.17: Normalized 2-ΔΔCT as fold expression level of DUR3 gene.

DUR3 was downregulated in boron treated wild type and BA8 cells whereas it was upregulated in absence of boron at BA8.

3.4 Whole Genome Transcriptomic (Microarray) Analysis

Microarray analysis was performed to compare up- and downregulated genes of BA8 with respect to the wild type under non-stress condition.

3.4.1 Determination of up/downregulated genes in BA8 compared to the wild type

cDNA microarray analysis was performed to investigate transcription profile of molecular mechanism for boron resistance in "BA8". This process included measuring expression levels of nearly 6000 genes in both wild type and BA8. *S. cerevisiae* CEN.PK 113.7D (905) was used as a reference strain to determine up/downregulated genes in BA8. During the microarray analysis YMM was used to

incubate both BA8 and wild type in non-stress condition and RNA samples were taken from the cultures at the exponential phase of growth by setting the cell concentration equal for both strains. The experiment was prepared from RNA samples with a RIN number higher than 8. Both up- and downregulated genes were accepted as meaningful according to expression change more than two fold in BA8 as compared to reference.

In the Table 3.5 some important upregulated genes which have more than 40-fold change and in Table 3.6 some highly downregulated genes that have more than 10-fold change and functions are given. Additionally genes which have 5-fold increase in mutant are indicated with systematic names acording to data intrepretation carrying out by using Funspec database.

Table 3.5: Genes whose expression was 40-fold higher or equal to that of wild type

Gene Name	Molecular Function
FMP43	Putative protein of unknown function; expression regulated by osmotic and alkaline stresses; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
HSP12	Plasma membrane localized protein that protects membranes from desiccation; induced by heat shock, oxidative stress, osmostress, stationary phase entry, glucose depletion, oleate and alcohol; regulated by the HOG and Ras-Pka pathways
HXK1	Hexokinase isoenzyme 1, a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; expression is highest during growth on non-glucose carbon sources; glucose-induced repression involves the hexokinase Hxk2p
STL1	Glycerol proton symporter of the plasma membrane, subject to glucose-induced inactivation, strongly but transiently induced when cells are subjected to osmotic shock

Table 3.6: Genes whose expression was 10-fold lower or equal to that of wild type

Gene Name	Molecular Function
PHO84	High-affinity inorganic phosphate (Pi) transporter and low-affinity manganese transporter; regulated by Pho4p and Spt7p; mutation confers resistance to arsenate
ZRT1	High-affinity zinc transporter of the plasma membrane, responsible for the majority of zinc uptake; transcription is induced under low-zinc conditions by the Zap1p transcription factor
ARO3	Catalyzes the first step in aromatic amino acid biosynthesis and is feedback-inhibited by phenylalanine or high concentration of tyrosine or tryptophan

The microarray analysis revealed that 956 genes were upregulated and 363 genes were downregulated above more than two fold change in BA8 compared to wild type (Data not shown). In Figure 3.18 and 3.19 the functional categories of upregulated and downregulated genes were analyzed by using Funcat (Ruepp *et al.*, 2004).

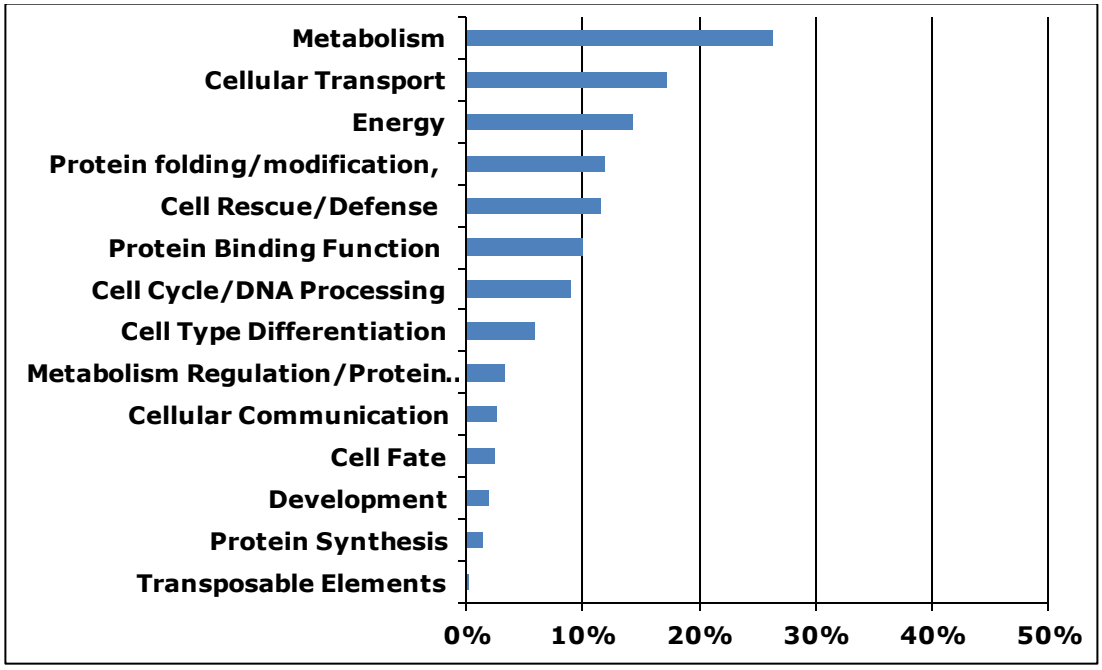

Figure 3.18: Functional categories of upregulated genes and ratio of these genes in related category (Ruepp et al., 2004).

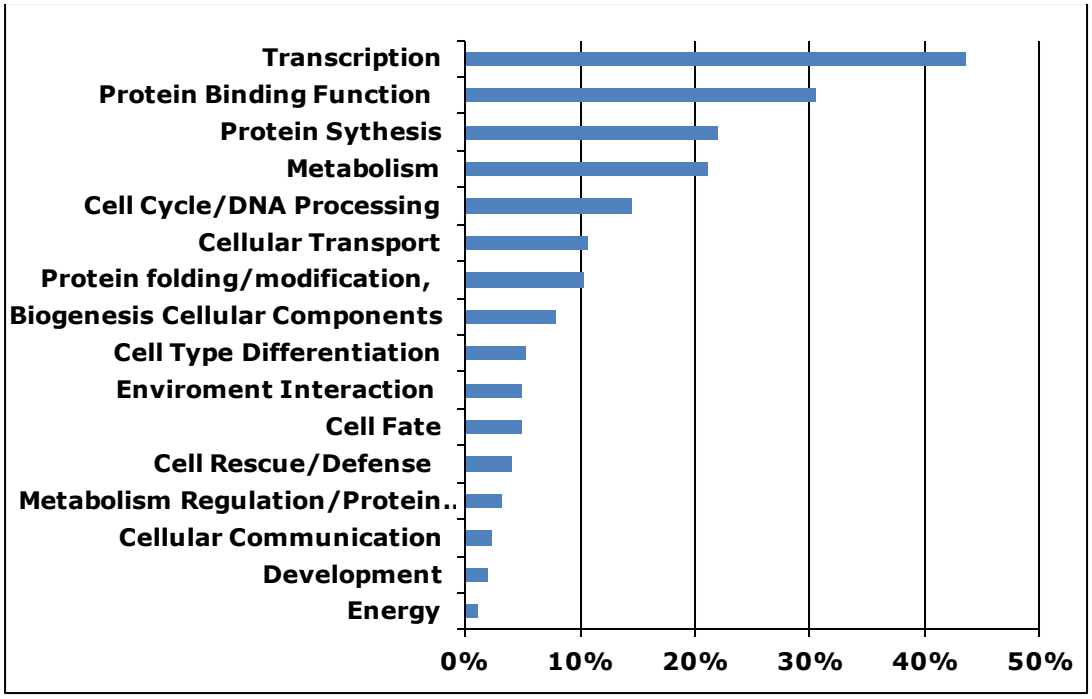

Figure 3.19: Functional categories of downregulated genes and ratio of these genes in related category (Ruepp et al., 2004).

Whole transcriptomic analaysis was demonstrated that according to upregulated and downregulated genes various pathways of *S.cerevisiae* were rearranged in boron resistant mutant strain. These pathways are shown in Figure 3.20, 3.21, 3.22, 3.23, 3.24, 3.25 and 3.26. Moreover pathway related genes are also shown in table 3.6, 3.7, 3.8, 3.9, 3.10, 3.11 and 3.12 with their up and down rate in BA8 and Wild type.

Table 3.7: Genes in BA8 and Wild type with their up and down regulation rate as related to Pentose Phosphate pathway

Gene Name	[BA8]	[wt]	Gene Name	[BA8]	[wt]
TKL2	2.13	-2.16	GND1	-0.04	0.10
SOL4	1.95	-2.11	RPE1	-0.06	0.03
GND2	1.24	-1.27	TAL1	-0.09	0.06
ZWF1	0.80	-0.36	TKL1	-0.13	0.17
SOL3	-0.01	0.03	RKI1	-0.58	0.65

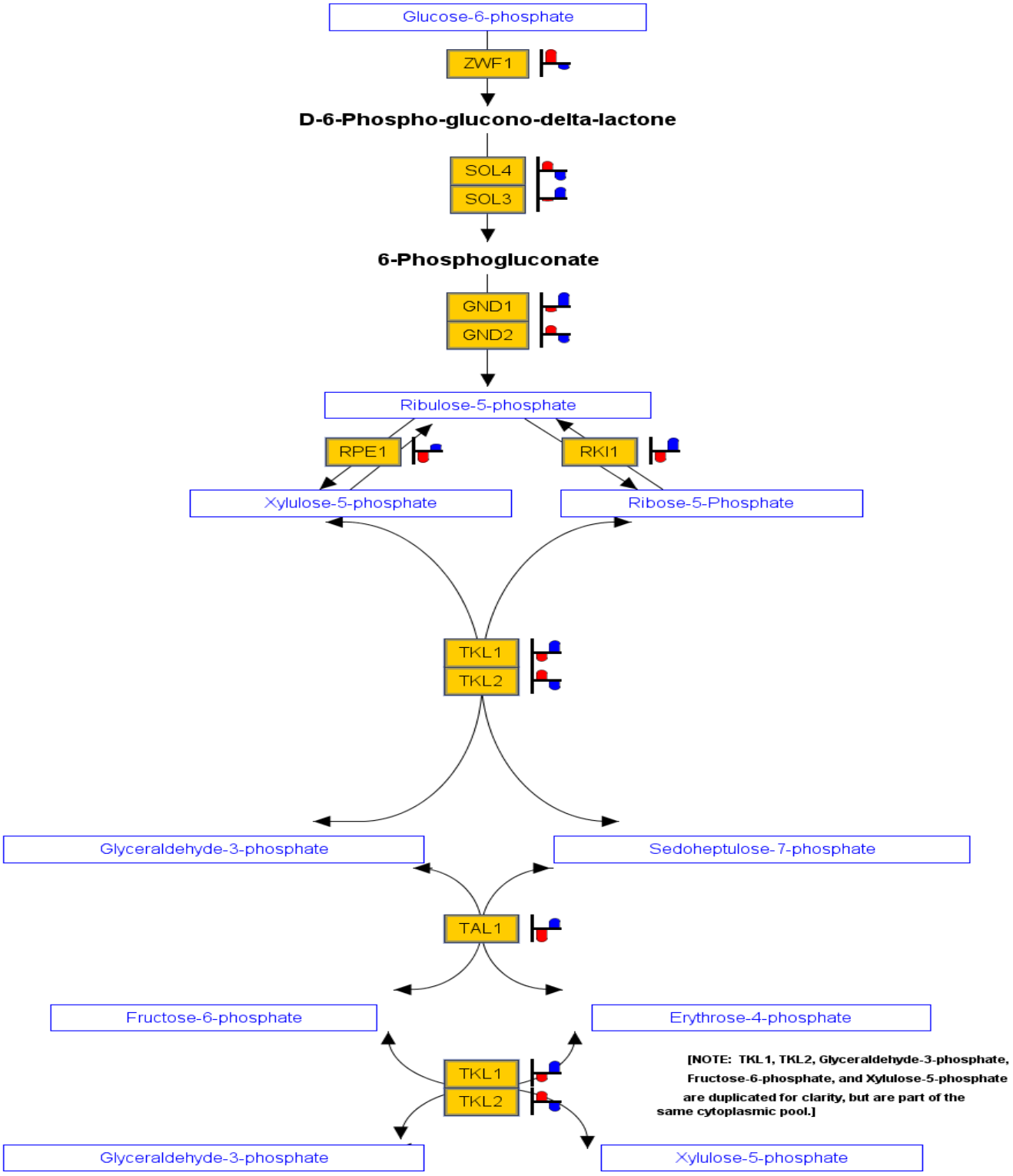

Figure 3.20: Pentose Phosphate pathway with related genes that have different fold change in BA8 and Wild type. A histogram graph being next to the most of gene names displayed up and down rate for each gene in BA8 (seem red) and wild type (seem blue).

It is known that glycolysis might be branched to pentose phosphate pathway to supply requirements of cells, but the result of microarray shown that BA8 upregulated some genes of pentose phosphate pathway (Table 3.6) to produce storage molecules as included high energy phosphate bonds (Figure 3.20). Gasch and co-workers (2000) stated that the stress conditions cause energy starvation because of the synthesis and utilization of response elements. Thus, cells needed cytoplasmic ATP source to supply high amount of energy demand. In order to hold steady ATP level in cytoplasm, cells direct all side pathways to main energy pathway to supply energy consumption.

Table 3.8: Genes in BA8 and Wild type with their up and down regulation rate as related to Tricarboxylic acid cycle (TCA) pathway. [Mt=Mitochondrion]

Gene Name	[BA8]	[wt]	Gene Name	[BA8]	[wt]
MDH2	1.06	-0.99	KGD1	0.19	-0.10
ICL1 (peroxisome)	1.00	-0.93	LAT1	0.19	-0.18
IDP2	0.96	-0.88	CIT3	0.14	-0.32
MLS1 (peroxisome)	0.94	-0.90	LPD1	0.11	-0.03
SDH1	0.83	-0.70	LSC2	0.09	-0.12
CIT1	0.83	-0.92	PDX1	0.06	-0.07
SDH3	0.59	-0.73	FUM1	0.04	-0.04
IDH1	0.49	-0.50	PDA1	0.04	-0.10
SDH4	0.47	-0.55	IDP1	0.02	0.04
SDH2	0.42	-0.38	LSC1	0.00	0.01
PYC2	0.36	-0.30	PYC1	-0.02	0.07
IDH2	0.34	-0.31	PDB1	-0.03	0.10
MDH3	0.33	-0.13	ACO1	-0.26	0.24
KGD2	0.33	-0.29	DAL7	-0.36	0.10
CIT2	0.29	-0.54	MAE1	-0.41	0.28
MDH1 (mt)	0.24	-0.37	ACO2	-0.44	0.37

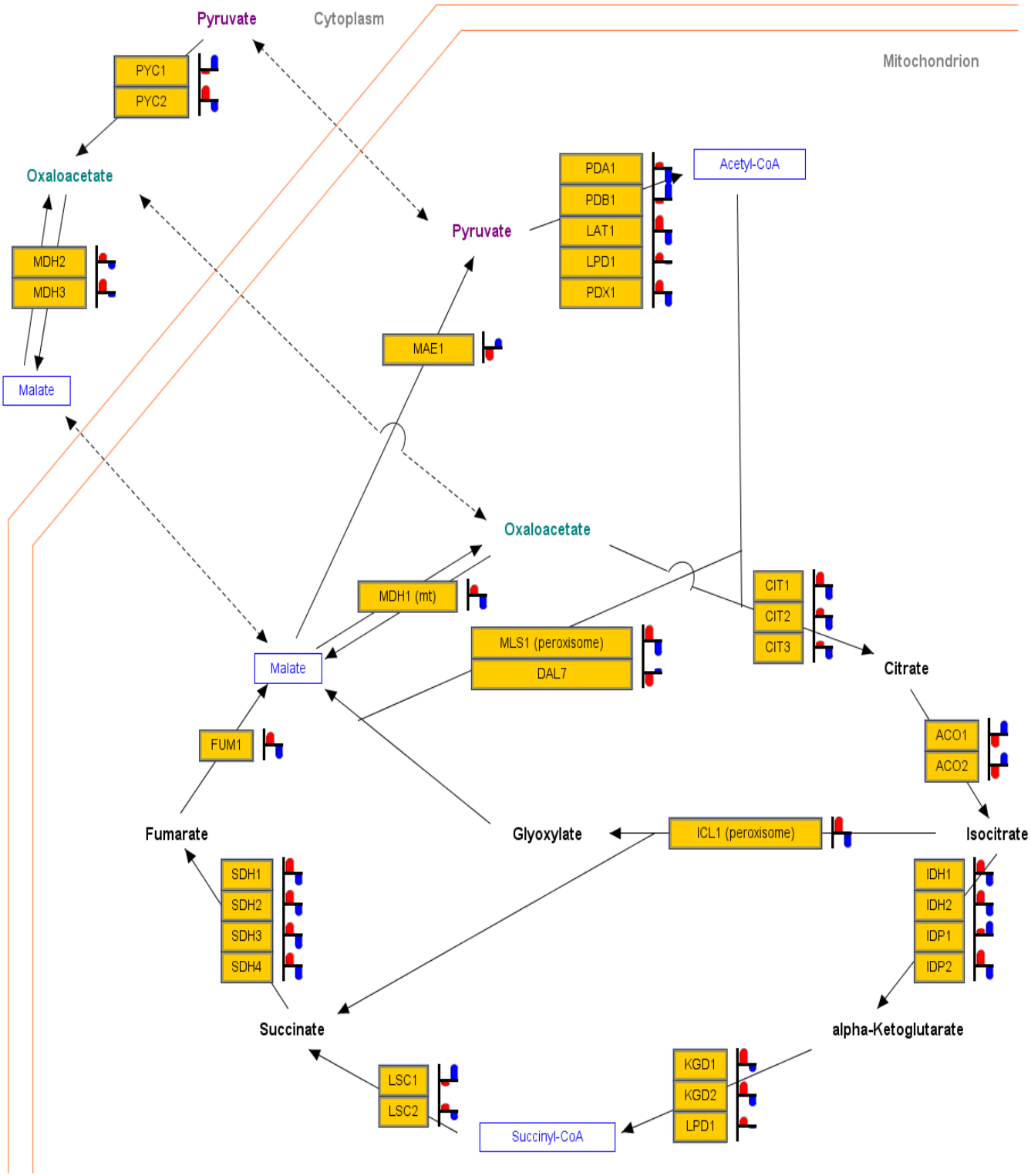

Figure 3.21: Tricarboxylic acid cycle (TCA) pathway with related genes that have different fold change in BA8 and Wild type. A histogram graph being next to the most of gene names displayed up and down rate for each gene in BA8 (seem red) and wild type (seem blue).

The energy starvation problem of cells rearranged the metabolism to the adaptation of high energy production. This is provided by upregulation of energy related genes as been responsible for ATP production in glycolysis and oxidative phosphorylation (Gasch *et al.*, 2000). TCA is essential pathway to regenerate ATP, because of that the results of microarray revealed that many genes of TCA were considerably upregulated in BA8 mutant in the Table 3.7. The genes' expression encoding the intermediate of TCA (NADH and $FADH_2$) were increased to generate ATP by oxidative phosphorylation (Figure 3.2).

Table 3.9 : Genes in BA8 and Wild type with their up and down regulation rate as related to Glycolysis and Gluconeogenesis pathway

Gene Name	[BA8]	[wt]	Gene Name	[BA8]	[wt]
HXK1	2.89	-2.87	CDC19	0.13	-0.33
MDH2	1.06	-0.99	PGI1	0.12	-0.11
PDC6	0.90	-1.00	ENO1	0.10	-0.31
FBP1	0.68	-0.46	TPI1	0.05	-0.07
PYK2	0.58	-0.58	ENO2	0.03	0.05
ALD6	0.58	-0.35	PGK1	0.02	-0.09
ADH5	0.43	-0.45	PYC1	-0.02	0.07
PDC1	0.40	-0.58	PFK1	-0.07	0.04
PYC2	0.36	-0.30	PFK2	-0.13	0.21
MDH3	0.33	-0.13	ACS2	-0.17	0.22
ADH1	0.28	-0.57	TDH2	-0.17	0.04
FBA1	0.26	-0.56	HXK2	-0.18	0.30
TDH1	0.21	-0.04	ADH2	-0.42	0.37
GPM1	0.20	-0.42	PDC5	-1.07	1.40
TDH3	0.18	-0.18			

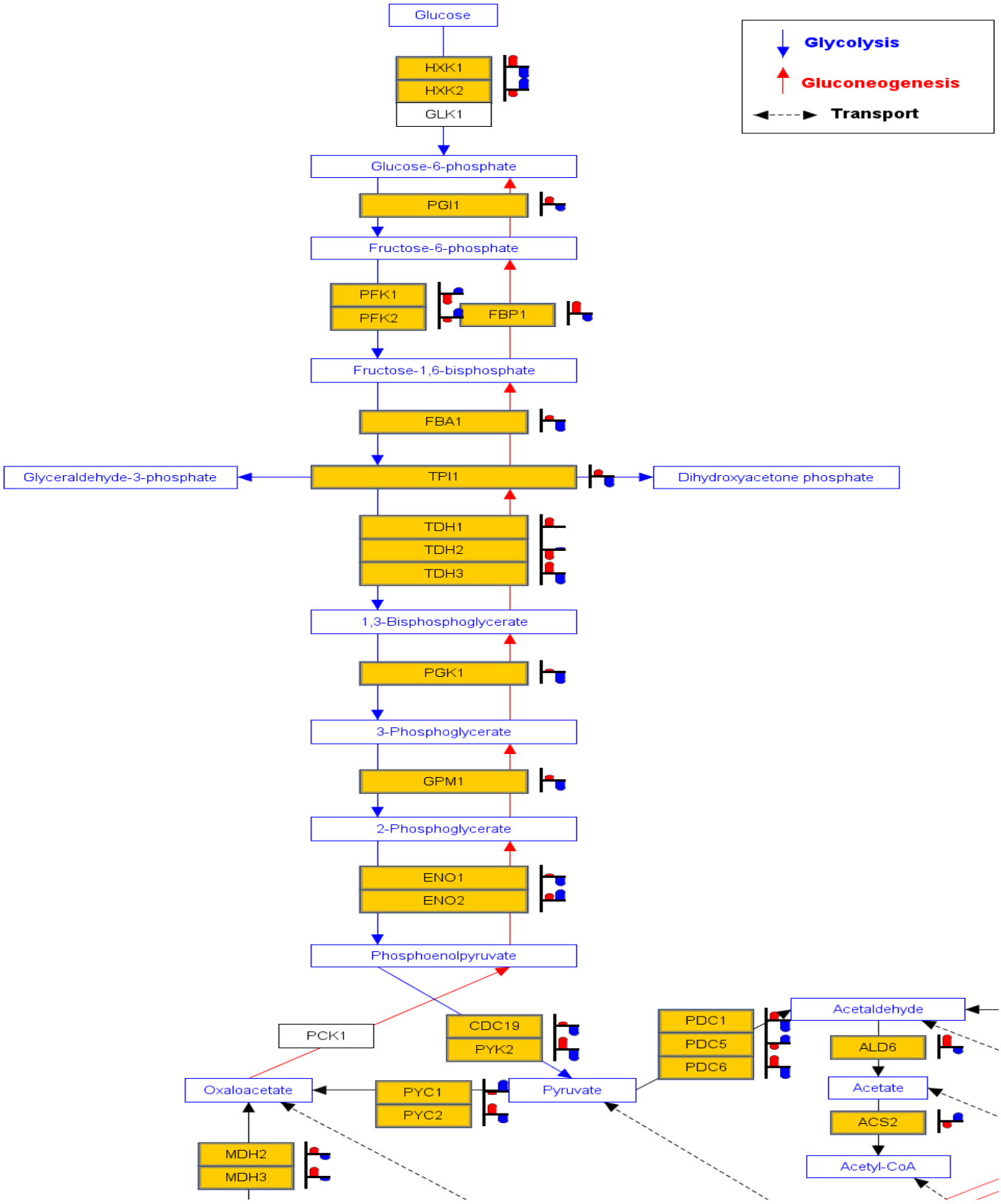

Figure 3.22: Glycolysis and Gluconeogenesis pathway with related genes that have different fold change in BA8 and Wild type. A histogram graph being next to the most of gene names displayed up and down rate for each gene in BA8 (seem red) and wild type (seem blue).

The glycolysis pathway is always main energy pathway for all organisms. Cells modulate glycolysis pathway in order to equilibrate the energy balance. The microarray analysis results shown that mostly induced glycolysis pathway might be a proof to present an example for energy starvation of BA8. BA8 mutant was obtained through stress selection, so the results of microarray in Figure 3.22 displayed that the genes of glycolysis pathway were highly upregulated to probably supply energy requirements. It was also observed that high number of genes as related to metabolism of glucose were upregulated in Table 3.8.

Table 3.10: Genes in BA8 and Wild type with their up and down regulation rate as related to Eukaryotic Transcription Initiation pathway

Gene Name	[BA8]	[wt]	Gene Name	[BA8]	[wt]
RPA135	-0.83	0.49	RPB7	-0.12	0.27
RPB5	-0.77	0.90	KIN28	-0.11	0.08
RPA190	-0.56	0.52	TOA2	-0.10	-0.01
TAF7	-0.55	0.46	RPB8	-0.10	0.20
RET1	-0.54	0.57	TAF12	-0.09	0.11
RAD3	-0.50	0.29	TFB4	-0.09	0.05
TFG2	-0.39	0.28	CCL1	-0.08	0.02
RPC19	-0.34	0.27	TAF6	-0.07	0.02
RPO26	-0.30	0.23	SPT15	-0.06	-0.04
RPB3	-0.23	0.18	SUA7	-0.05	0.15
RPC11	-0.20	0.36	RPB2	-0.03	0.03
TFB2	-0.18	0.15	TFB3	-0.02	-0.01
RPC25	-0.18	0.04	TAF9	0.13	-0.09
RPO21	-0.18	0.15	SSL2	0.18	0.02
TAF5	-0.15	0.17	RPB11	0.25	-0.20
TAF13	-0.15	0.23			

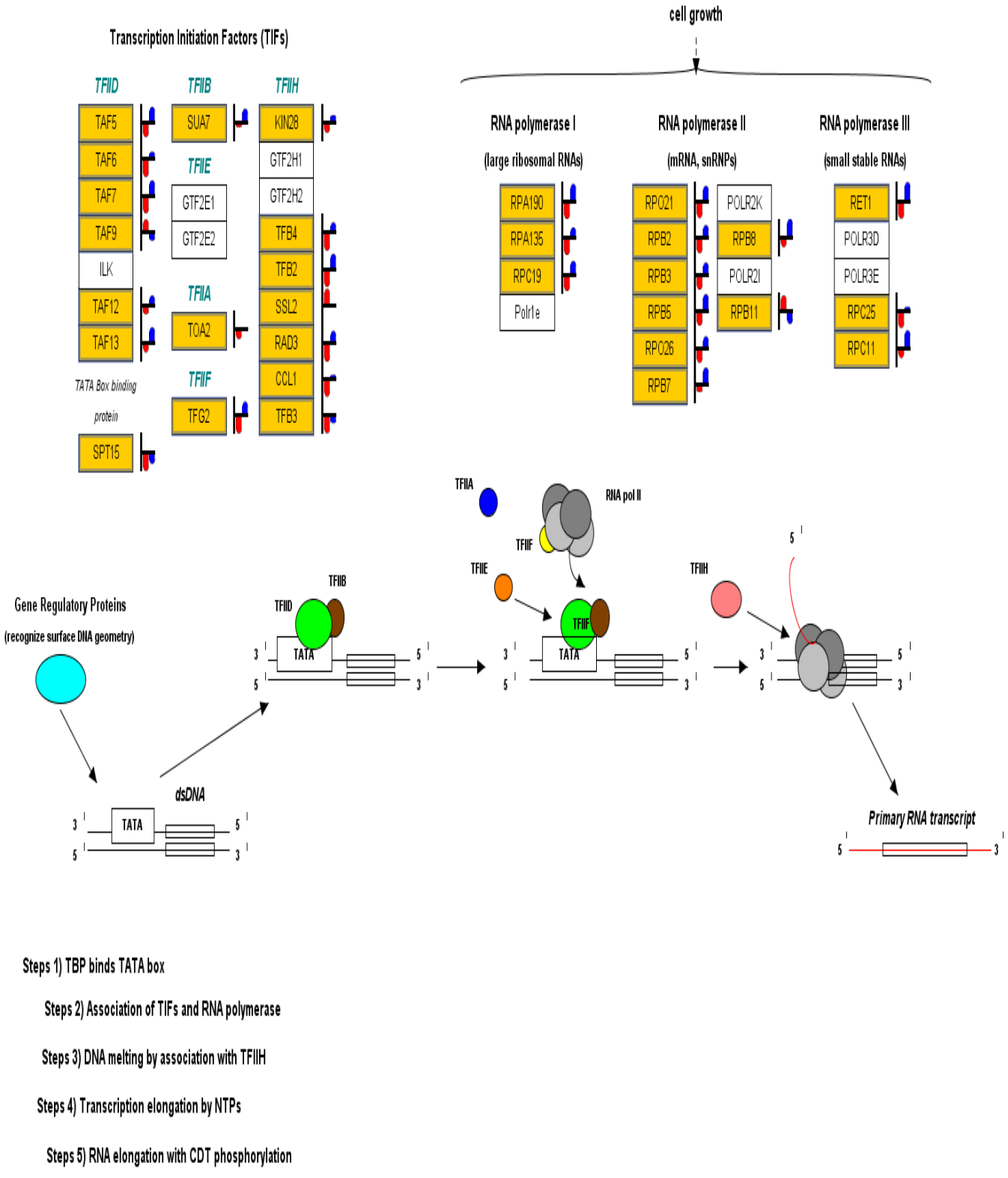

Figure 3.23: Eukaryotic Transcription Initiation pathway with related genes that have different fold change in BA8 and Wild type. A histogram graph being next to the most of gene names displayed up and down rate for each gene in BA8 (seem red) and wild type (seem blue)

Transcription metabolism is crucial for all organism, but it needs high amounts of energy. Therefore, it was thought that BA8 might be decreased transcription level to prevent energy consumptions. The results of microarray also supported those hypothesis. Stress conditions caused reduction of genes encoding rRNA and ribosomal proteins to supply sufficient energy with inhibition some pathway that requires substantial energy and cellular mass, because of that protein synthesis and transcription metabolism were downregulated (Figure 3.23).

Table 3,11: Genes in BA8 and Wild type with their up and down regulation rate as related to Proteasome Degradation pathway

Gene Name	[BA8]	[wt]	Gene Name	[BA8]	[wt]
UBI4	1.08	-0.92	PRE10	0.03	-0.08
UBC5	0.40	-0.49	RPN6	0.01	-0.01
RPN10	0.37	-0.13	RPN3	-0.05	0.11
RPN8	0.36	-0.28	PRE6	-0.06	0.05
RPN12	0.35	-0.19	RPT3	-0.08	0.08
NAS2	0.33	-0.27	HTA2	-0.10	0.12
UBC4	0.30	-0.34	PRE5	-0.10	0.02
RPN2	0.23	-0.16	PRE2	-0.12	0.09
PRE9	0.21	-0.21	RPN9	-0.13	0.28
PRE8	0.20	-0.30	RPT1	-0.13	0.05
PRE3	0.20	-0.16	RPT5	-0.16	0.18
HTZ1	0.14	-0.13	PUP2	-0.17	0.23
UBA1	0.12	-0.08	RPN5	-0.18	0.29
NAS6	0.11	-0.15	PRE1	-0.19	0.18
PRE4	0.10	-0.17	RPT2	-0.24	0.29
PUP1	0.06	-0.01	RPT6	-0.28	0.22
SCL1	0.04	-0.08	PRE7	-0.32	0.30

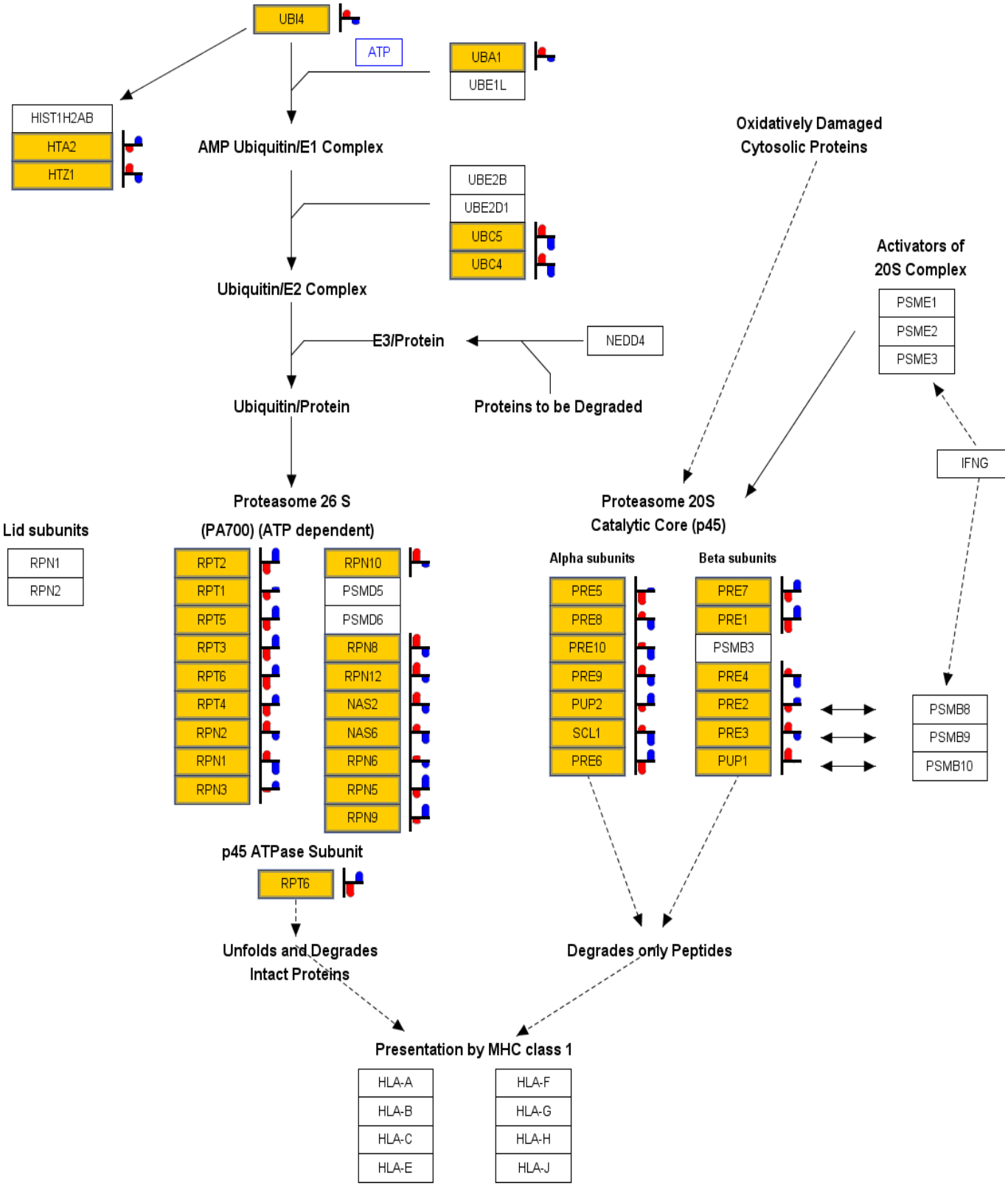

Figure 3.24: Proteasome Degradation pathway with related genes that have different fold change in BA8 and Wild type. A histogram graph being next to the most of gene names displayed up and down rate for each gene in BA8 (seem red) and wild type (seem blue).

It is known that protein syhthesis consumed much more energy, but proteasome degradation provides internal compounds as used in energy production pathways. In Figure 3.24 it is seem that the genes associated with ubiquitination and degradation of proteins were highly upregulated in BA8 compared to wild type. It might be suggested that the mutant cells were aimed to prevent energy starvation by upregulated proteasome degradation pathway to both reduce energy using by ribosome synthesis and also create a new sources for energy pathway to convert ATP.

Table 3.12: Genes in BA8 and Wild type with their up and down regulation rate as related to Cholestrol Biosynthesis pathway

Gene Name	**[BA8]**	**[wt]**	**Gene Name**	**[BA8]**	**[wt]**
ERG20	-1.33	1.19	ERG25	-0.24	0.24
ERG1	-0.46	0.48	ERG13	-0.21	0.09
ERG11	-0.42	0.32	ERG7	-0.07	0.03
ERG3	-0.38	0.32	IDI1	-0.06	-0.02
MVD1	-0.35	0.04	ERG26	-0.03	0.04
HMG1	-0.33	0.35	ERG9	0.06	-0.05
ERG12	-0.32	0.31			

It is very commonly known that a number of organism lastly use fatty acids as a source of energy rather than proteins and carbohydrates. However, when cells are hungry for energy, they rearrange fatty acid pathway to supply required acetil coA as been crucial for TCA that is a center of oxidative phosphorylation by generating ATPs and ATP production compounds. The Figure 3.25 and Table 3.11 as displayed downregulated genes that are associated with acetil coA convertion to fatty acid, also support the presuppositions.

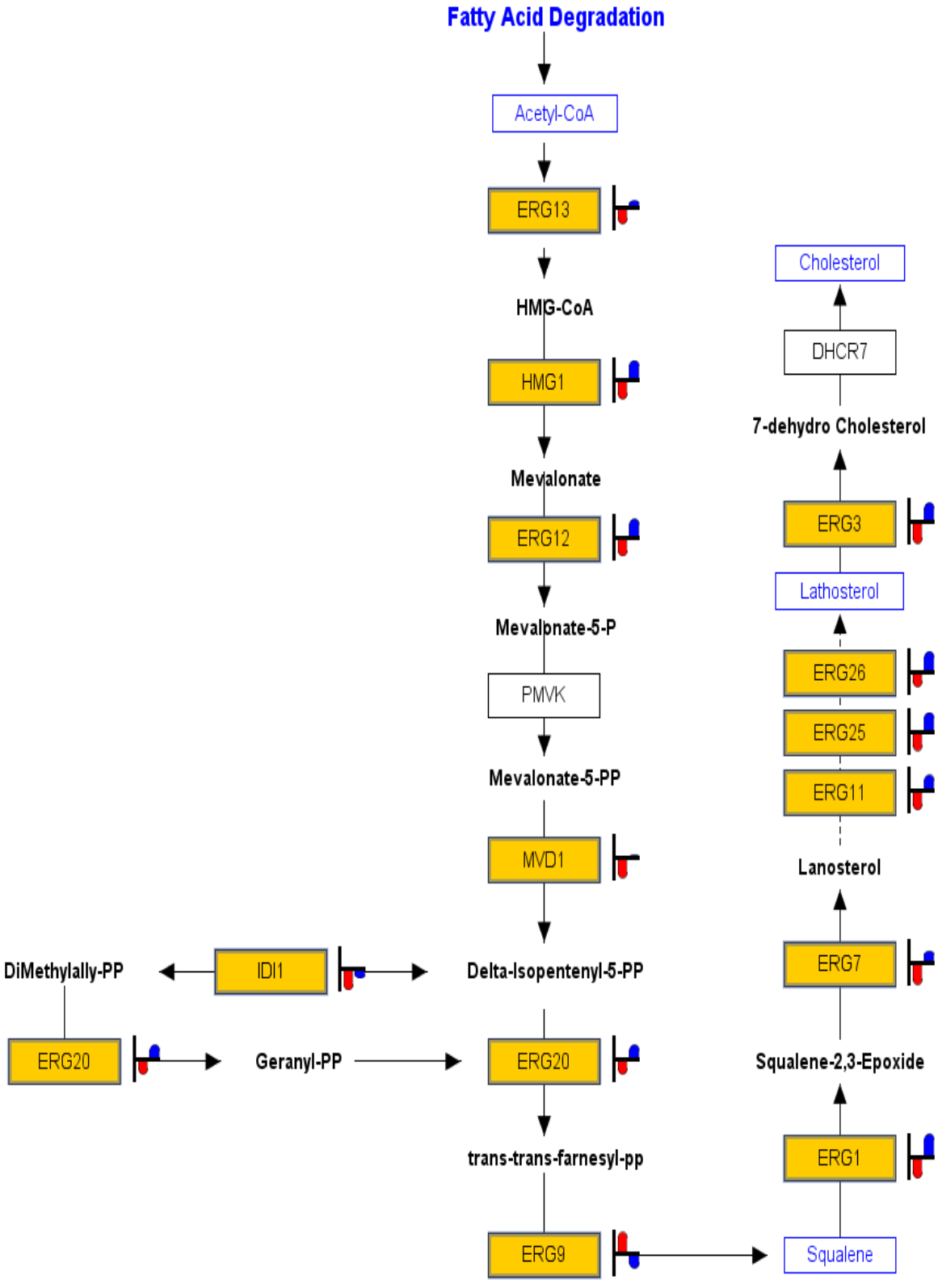

Figure 3.25: Cholesterol Biosynthesis pathway with related genes that have different fold change in BA8 and Wild type. A histogram graph being next to the most of gene names displayed up and down rate for each gene in BA8 (seem red) and wild type (seem blue)

Table 3.13: Genes in BA8 and Wild type with their up and down regulation rate as related to Heme Biosynthesis pathway

Gene Name	[BA8]	[wt]	Gene Name	[BA8]	[wt]
HEM12	-0.66	0.71	HEM14	0.11	-0.04
HEM3	-0.24	0.20	HEM2	0.12	-0.15
HEM1	-0.07	0.03	HEM13	0.18	-0.18
HEM4	0.04	0.01	HEM15	0.21	-0.04

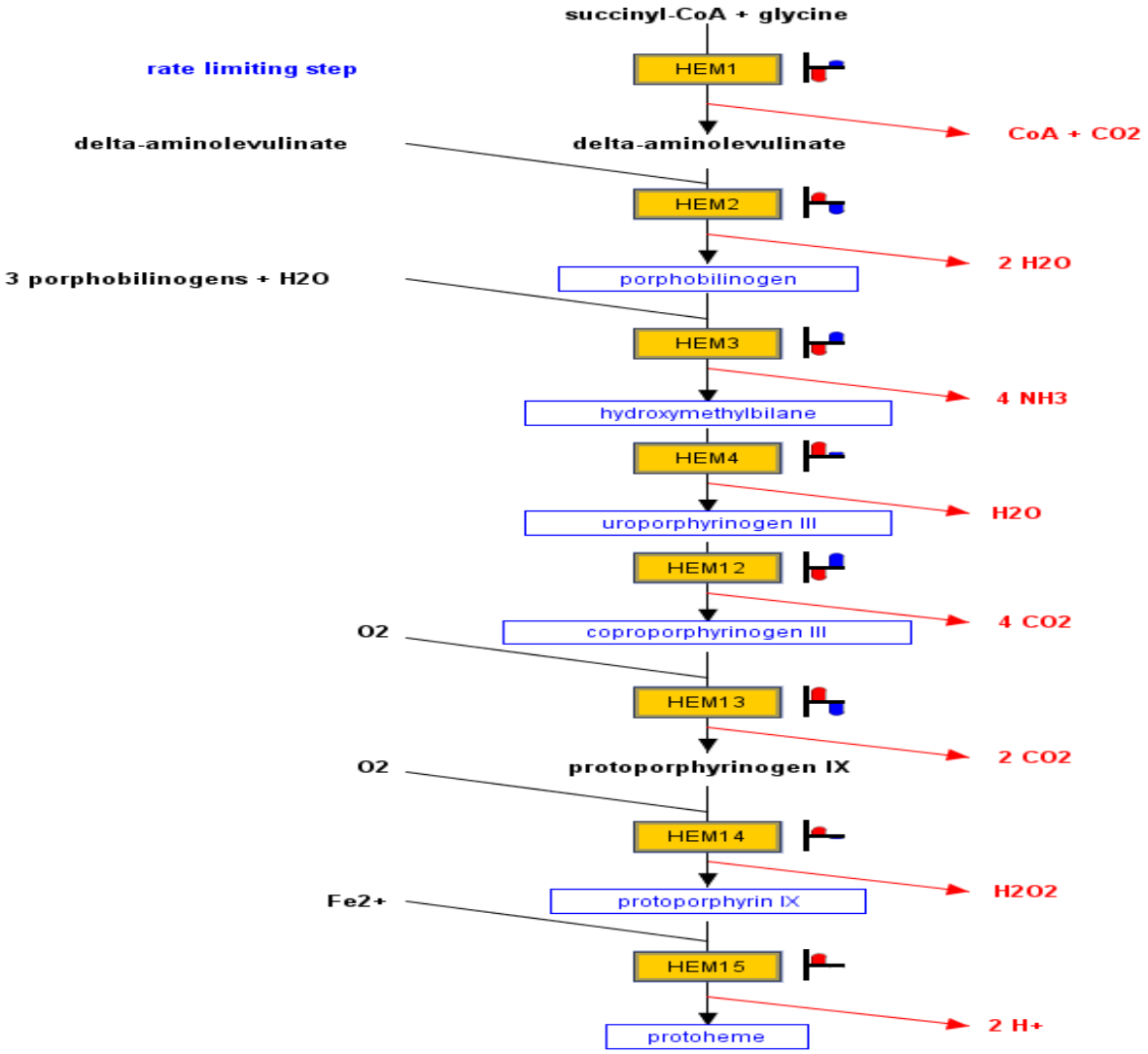

Figure 3.26: Heme Biosynthesis pathway with related genes that have different fold change in BA8 and Wild type. A histogram graph being next to the most of gene names displayed up and down rate for each gene in BA8 (seem red) and wild type (seem blue).

The pathway results of microarray as demonstrated in Figure 3.26 revealed that BA8 mutant seemed to conserve acetil-coA and succinyl-coA molecules to use predominantly in energy production thus downregulated genes related to succinyl-coA reaction in heme biosynthesis but also upregulated genes associated with electron transport redox reactions that provided energy flux to generate ATPs.

3.5 Comparision of Microarray and qRT-PCR Results for *BOR1, ATR1* and *DUR3*

It was previously mentioned that qRT-PCR was used to determine expresion levels of boron related genes; *BOR1, ATR1* and *DUR3* with RNA isolated at three OD600. However microarray analysis was performed at OD600=1, because of that the expression levels of *BOR1, ATR1* and *DUR3* genes was investigated at OD600=1 to compare both results each other (Figure 3.27 and Table 3.13).

Table 3 14: Expression of BOR1, ATR1 and DUR3 genes in microarray analysis and qRT PCR

Gene Name	Microarray Analysis	qRT-PCR Analysis
ATR1	-1.8	0.6
BOR1	1.36	1.4
DUR3	2.03	1.8

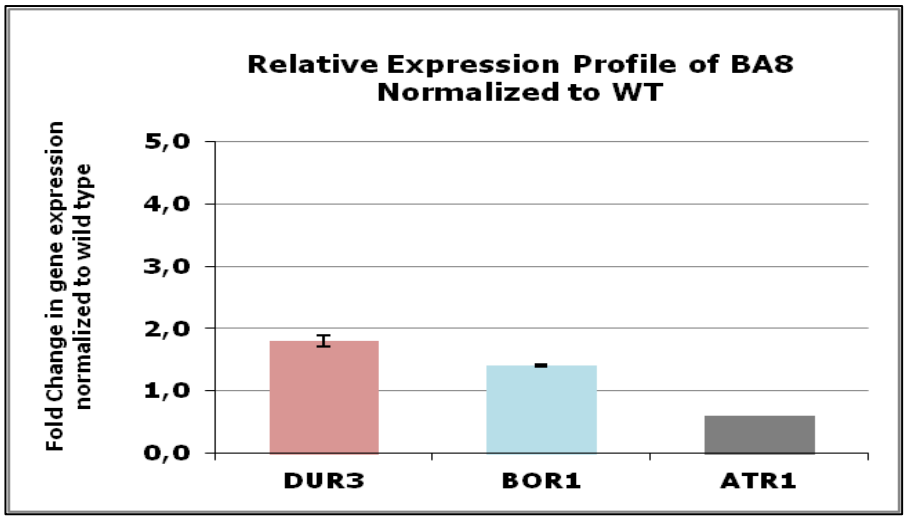

Figure 3.27: QRT-PCR was performed for wild type and BA8 to determine the expression level differences of ATR1, BOR1 and DUR3 genes that are involved in boron transport mechanism.

4. DISCUSSION AND CONCLUSIONS

The aim of this study was to characterize molecular mechanism of boron resistance by using a *S.cerevisiae* mutant "BA8" as previously obtained via evolutionary engineering by Dr. Ulkü Yılmaz (MOBGAM-Istanbul, Istanbul Technical University). In this context, the cross-resistance phenotype, metabolite consumption and production profiles and gene expression profiles were investigated by means of different methodologies.

The comparison of boron resistant strain (BA8) to wild type displayed that it has high capability to tolerate boron. Thus far, there did not exist a boron-resistant mutants obtained by evolutionary engineering in the literature soBA8 might be crucial to clarify boron resistance mechanisms in yeast.Mutant individual BA8 and wild-type were inoculated on solid media containing YMM without stress as control one, 0.5 mM $ZnCl_2$, 0.5 mM $NiCl_2$, 20 mM $FeCl_3$, 3 mM $CrCl_3$, 3 mM $CoCl_2$ and 10 % NaCl, after serially diluted in a range between 10^0-10^{-5}.

The mutant strain BA8 showed cross-resistance to nickel, chromium and cobalt stresses very slightly, when compared to wild-type. There is no significant cross-resistance of boron-resistant mutant strain to investigated metal stress conditions. At 10 % NaCl (w v^{-1}) salt stress condition, BA8 was not found to gain cross-resistance to osmotic stress. However, there is no common phenotype related to boron resistance.

Kaya et al. (2009) stated that upregulation of *ATR1* leads to salt tolerance beside boron resistance. However, this correlation did not observed in boron-resistant mutant individual BA8. On the other hand, mutant individual BA8 had a less resistance against 5 % (v/v) ethanol stress. Consequently, boron resistant mutant strain BA8 was become prominent with salt and ethanol cross-resistances, respectively.

Nozawa et al. (2006) stated that boron treatment changes the lipid composition of the cellular membranes and eventually boric acid stress change the membrane

permeability (Nozawa *et al.* 2006) and ethanol stress tolerance also changes the plasma membrane composition (Lei et al. 2007).

The boron-resistant strain BA8 and wild type were grown together in presence and absence of boron to perform growth curve analysis. The OD_{600} measurements shown that 100 mM $B(OH)_3$ considerably inhibited the growth of wild type cultures whereas it has no effect on the growth performance of BA8. This question was answered by M. Schmidt (2010) that overdose of Boric Acid damages cytoskeleton at the bud neck that cause irregular septa and bring with synthesis of aberrant cell wall protuberances. However in the boron free media, the μ_{max} and OD_{600} values displayed that wild type could grow better than BA8. It can be explained by non-ideal genome modifications of mutagenized culture cells that did not cope with adaptation problem. Furthermore; the results of cell dry weight (Figure 3.10 and 3.11) were in parallel to growth curve analysis (Figure 3.5). BA8 and wild type arrived early to stationary phase during absence of boron when stress groups went on to growth.

On the other hand there were not any significant differences between BA8 and the wild type, regarding growth and metabolite production as tested by HPLC. However both of wild type and BA8 produced glycerol in either presence or absence of boron condition (Reed, 1987). It is known that glycerol is protector material to response in stress cases. Glycerol production was similar with glucose consumption. Highest glycerol concentrations of the medium were close to each other.

The ethanol production of wild type and BA8 strains seems to be parallel to glucose consumption. When glucose depleted in the medium, ethanol began to decrease. It was demonstrated that all of strains included mutant could utilized ethanol to grown on as a carbon source. This situation might be explained by claiming that BA8 could use carbon source from outside after glucose depletion.

Some of organisms produce important reserved carbohydrates such as trehalose and glycogen to avoid negative effects of general stress (François and Parrou, 2001). To this end, the trehalose and glycogen contents of wild type and BA8 in presence and absence of boron medium were measured by enzymatic assay. Trehalose and glycogen production profiles in both absence and presence of boron could not change for BA8 mutant rather than wild type as accumulated trehalose and glycogen in

presence of boron condition. The accumulation of trehalose and glycogen contents without boron stress in BA8 mutant might be expressed by having a defect to detect stress in medium, thus it hold reserved carbohydrates metabolism as active mode. In 2005 Jules and his group explained that trehalose accumulation in the cells might be caused by toxic effect of metal stress on the proteins and lipid membrane via Fenton reaction since trehalose functions as antioxidant and protects cell integrity from lipid peroxidation in addition to maintaining proteins in their native conformation cooperating with molecular chaperons. It was observed that BA8 and wild type gave up accumulating trehalose after glucose depletion in the medium to use them as a carbon source. Moreover the microarray result shown that genes related to trehalose metabolism and glycogen biosynthetic process was highly upregulated.

The expression level differences of *ATR1, BOR1 and DUR3* genes as determined boron related from literature was shown by qRT-PCR analysis.

The expression profile of *BOR1* in wild-type and *BA8* were analyzed and investigated under boron stress conditions. *BOR1* gene is homolog to the NaBCI mammalian electrogenic Na1-coupled borate transporter which has a function on boron homeostasis in mammals (Park et al. 2004). *BOR1* is the first characterized boron tolerance gene in *S. cerevisiae*. It is a boric acid/borate efflux transporter. Overexpression of *BOR1* reduced boron accumulation and leads to boron resistance in yeast (Takano et al. 2008; Kaya et al. 2009). However, it is interesting that the expression level of *BOR1* at high boron concentrations is only slightly increased in contrast with *ATR1,* due to boron limited conditions (Takano et al., 2007).The expression level of *BOR1* as also defined encoding localized plasma membrane protein; Bor1p that exports excess boron out of the cell to maintain ionic homeostasis inside the cell (Sá-Correia et al., 2008), but this export based on storage boron inside cell. However first investigation of *BOR1*was revealed that *A. thaliana* needs boron for its developmental stage, so its roots consist of AtBOR1 protein involving in B xylem loading. During the absence of B; AtBOR1 is highly expressed; but when plant exposes to high B content AtBOR1 is degraded by post-transcriptional regulation (Takano *et al.,* 2005). On the Figure 3.13 seem that BA8 mutant in absence of boron expressed *BOR1* at very high level when compared to BA8 and wild type as in presence of boron. Takano mentioned about as degradation of Bor1p or transcription product of *BOR1* that Takano mentioned about in 2005 for plant

might be reason of decreased *BOR1* expression in stress condition. It was also thought that BA8 mutant could behave as if in presence of boron even if in absence of boron.

ATR1 is a drug-H^+ antiporter in DHA2 family and confers resistance to histidine homologue aminotriazole drug (not found in nature and leads histidine limitation state) in yeast (Kanazawa et al. 1988; Sa-Correia et al. 2008). The novel discovered function of *ATR1* in boron resistance indicated that it was the main efflux pump responsible for boron tolerance (Kaya et al., 2009), so its expression level on boron-resistant mutant individual BA8 was tested. The relative expression profiles of wild-type and BA8 was investigated. In the mutant individual BA8, relative fold change in *ATR1* expression was 2-fold increased when exposed to 80 mmol l^{-1} boric acid stress concentration compared to wild type. At the same conditions wild-type had only 1.2 fold of increase and had a limited *ATR1* expression. On the other hand, mutant individual BA8 had a remarkable 3.4-fold of increase without boron stress exposure (Figure 3.14). Similar to *BOR1*, expression profiles of *ATR1* at control conditions was increased. Curiously, the 3.4-fold upregulation at normal physiological conditions was higher of wild-type's 1.4 fold of increase at boric acid stress related physiological condition.

Otherwise the expression pattern of *DUR3* in BA8 mutant and wild type were analyzed in absence and presence of boron. Dur3 transporter protein is also an efflux transporter like Bor1 and Atr1, but it has differences from them to import boron inside cells (Jennings *et al.*, 2007 and Takano *et al.*, 2005). Although its functions are not clarified yet, over expression of *DUR3* increased boron levels inside the cells (Nozawa *et al.*, 2006). It can also suggested that *DUR3* has a role to uptake boron inside the cell, and work against to *ATR1* and *BOR1* acting to export boron to provide homeostasis. The experiment of Nozawa group in 2006 demonstrated that dur3Δ mutant can live easily under the toxic boron condition, but it is not valid for *DUR3* plasmid consisted mutant that cannot survive during high level boron concentration. As a result of that the expression level of *DUR3* as analyzed by qRT-PCR in wild type and BA8 mutant decreased under the 1-fold in the presence of boron, when it increased to above the 8-fold in BA8 mutant in the absence of boron.

In this study, DNA microarray analysis was performed to observe transcriptomic differences between boron resistant strain and wild type.

The results of DNA microarray analysis displayed that a lot of genes were upregulated and downregulated in boron resistant mutant "BA8" in the absence of boron condition. It can be explained that when 2.0 fold change was selected as bottom limit of expressed genes, there were 956 upregulated and 363 downregulated genes for BA8. In 2000, Gasch and his co-workers also revealed that approximate 900 genes in "environmental stress response" (ESR) might be changed at whole genome after stress exposure. ESR genes can be affected by most of stress condition with leading transiently large expression change. In spite of absence of stress condition, microarray analyses of BA8 showed parallel results of transcription profile as seen during ESR. It is claimed that BA8 might be ready for external stress conditions that could partly explain its boron resistance in any moment, because of being under ESR state without stress condition.

The results of DNA microarray analysis in Figure 3.16 demonstrated the relation of 963 up regulated genes with pathways of *S.cerevisiae*. The biggest rate "26.4%" of these genes were related to metabolism and highly expressed of them was responsible for C-compound and carbohydrate metabolism. The second high number of up-regulated genes had been in cellular transport pathway as carried especially carbon source to provide required energy suppliers. The other most active pathway with 14.7% rate was energy metabolism in terms of including considerably expressed genes.

The biggest contribution of up regulated genes to carbohydrate metabolism might be similarly seen in environmental stress response. It was clarified that HSP's or resembling resistance mechanism genes were upregulated in ESR when cells exposed to stress condition by Ogawa and his friends in 2000. In this case cells needed producing cytoplasmic ATP source to supply high amount of energy as depleted by synthesis and utilization of response elements.

Because of that cells were affected by energy starvation to keep ATP levels as stable in their cytoplasm. The main adaptation against this drawback is induction of energy production pathways, such as glycolysis and oxidative phosphorylation (Gasch *et al.*, 2000).

The microarray analysis results shown that mostly induced glycolysis pathway might be a proof to present an example for energy starvation of BA8. It was also observed

that high number of genes as related to metabolism of glucose were upregulated in Table 3.8. These genes consisted of a hexokinase that catalyzes the first step in glycolysis and responsible for priming reaction (*HXK1*), cytoplasmic malate dehydrogenase, one of three isozymes that catalyze interconversion of malate and oxaloacetate (*MDH2*) and minor isoform of decarboxylase (*PDC6*) that decarboxylates pyruvate to acetaldehyde, involved in amino acid catabolism and is strongly induced during sulfur limitation. Besides this some gene group being in glycolysis were also highly up-regulated such as *ALD3, ALD4, ALD6* that related to aldehyde dehydrogenases and hexose import genes (*HXT6, HXT5, HXT15, HXT16, HXT7*).

The boron resistant strain fairly activated various pathway to supply energy as required such as regulation of glycolysis and gluconeogenesis, electron transport and membrane-associated energy conservation, respiration especially aerobic form, metabolism of energy reserves (e.g. glycogen, trehalose), oxidation of fatty acids and energy conversion with regeneration or newly production (e.g ATP synthase). The mostly induced genes were in electron transport chain various subunits of cytochrome c oxidase (*COX1, COX2, COX3, COX7, COX13, COX14, COX5B* and *COX20*), and genes responsible in ubiquinone (Coenzyme Q) biosynthesis (*COQ4* and *COQ9*). The same genes were also over-expressed under ESR (Gasch *et al.*, 2002). It was explained that necessary of high amount of energy caused to continuously mitochondrial activity were provided from that genes encoding subunits of ATP synthase (*ATP6, ATP15, ATP18, ATP19).* It can be assumed that upregulation of these genes as associated with oxidative phosphorylation might be also provided tolerance to oxidative stress.

The energy requirements depending on glucose catabolism of BA8 might be explained by ESR-related protection results in increased energy consumption (Gasch *et al.* 2002). Therefore it can be implied that cells upregulated catabolic pathways to balance energy depletion. It was observed that BA8 had not serious growth problem under the control condition despite highly induced transcription profile. In order to express this situation the highly activated glycolysis and oxidative phosphorylation might be a result of satisfying to extended interval of energy.

On the other hand; although the other carbon sources were repressed by glucose, genes associated with maltose metabolism as controlled by maltose transporter

(*MAL11, MAL31*) and maltases (*MAL12, MAL32*) and galactose consumption pathway including catalyzer of interconversion of UDP-galactose and UDP-D-glucose (*GAL10*), galactose permease (*GAL2*) and DNA-binding transcription factor required for activating GAL genes (*GAL4*) were over-expressed. Moreover there was a highly upregulated key gene; *GRE* encoding aldose reductase involved in methylglyoxal, d-xylose, arabinose, and galactose metabolism; induced by osmotic, ionic, oxidative, heat shock, starvation and heavy metals stress and also regulated by the HOG pathway. It can be expected that BA8 has ability to use alternative carbon sources such as maltose and galactose nevertheless glucose as caused catabolite repression against other carbon sources during presence of itself in yeast cells (Federoff *et al.*, 1983).

Besides considerably triggered energy regeneration, BA8 also mostly activated genes that related to proteasome degradation to expose manomers as used for energy pathways. It was also known that protein synthesis and translation metabolism especially ribosome production required high amount of energy. Because of that cells upregulated genes associated with ubiquitination process in which proteins were degraded such as *UBI4* that becomes conjugated to proteins to mark them for selective degradation via the ubiquitin-26S proteasome system.

That is essential for the cellular stress response and encodes as a polyubiquitin precursor comprised of 5 head-to-tail repeats and *UBC8* as called ubiquitin-conjugating enzyme that negatively regulates gluconeogenesis by mediating the glucose-induced ubiquitination of cytoplasmic enzyme, fructose-1,6-bisphosphatase (FBPase) that catalyzes the ubiquitination of histones in vitro.

It was previously mentioned that BA8 over-expressed genes has responsible for aerobic respiration pathway in spite of growing on anaerobic condition. Most of these genes related to reactive oxgen species (ROS) protection? The cytosolic superoxide dismutase (*SOD2*) and cytosolic catalase (*CTT1*), which degrade superoxide and hydrogen peroxide were some of them. Furthermore, *GPX1* encoding glutathione peroxidase uses organic peroxides as substrate similar to catalase specifically reduces hydrogen peroxide was another upregulated gene in ROS exposure.

In 2000 Gasch *et al.*, defined that diverse stress conditions cause a general response from ESR as rearranged by each environment. They also said that it might be observed a specialized and unique response except general stress responses during various stresses. According to results of this study widely induced aldehyde dehydrogenase gene (*ALD3)* which has capability to conserve toxic acetaldehyde compounds to less toxic carboxylic acid forms was one of the special responses for boron resistant strain. ALDHs are controlled of this reaction as occurred in both mitochondria and the cytosol by encoding *ALD6*, *ALD2* and *ALD3* in cytosol and ALD4 and *ALD5* in mitochondria (Navarro-Aviño *et al.*, 1997).

Otherwise the downregulated genes analysis of BA8 demonstrated that protein synthesis and transcription pathways were significantly repressed. Around 95% of 363 down-regulated genes also being in more than one pathways were associated with nucleotide/nucleoside/nucleobase metabolism, rRNA and tRNA synthesis, RNA processing, RNA modification, ribosome biogenesis, translation initiation and nucleic acid/RNA/ATP binding. Once again Gasch *et al.*, revealed that ESR had displayed same regulations in presence of stress substances in 2000. This state was clarified by McAlister and Finkelstein in 1980 as claiming that cellular translation was declined to response stressful environmental transitions.

Stress conditions caused reduction of genes encoding rRNA and ribosomal proteins to supply sufficient energy with inhibition some pathway that requires substantial energy and cellular mass, because of that protein synthesis and transcription metabolism were downregulated. There are some ideas to explain this case in literature; the first one was performed by Eisen *et al.*, in 1998 as resulted with yeast genome have various strictly co-regulated genes and the ribosomal protein genes are some of them. In addition to that it was claimed that environmental stress conditions lead to sudden fall at expression of the ribosomal protein genes (Causton *et al.*, 2001). Apart from these; a recent study reflected that boron damaged to protein synthesis by upgrading phosphorylation of eIF2a in a Gcn2 kinase dependent manner. Gcn2 that also called uncharged tRNA binding domain (HisRS) requires to phosphorylate eIF2a under the boron condition. It can be implied that boron toxically affects activation of the general amino acid control system so that cause to inhibition of protein synthesis.

It is known that the general amino acid control pathway is conserved among eukaryotes, so this mechanism of boron toxicity might be of essential (*Uluisik et al.*, 2011).

The results of BA8 microarray analysis represented that most of genes involved in nucleic acid and RNA/protein productions, modulations and regulations were deeply downregulated. For instance Phosphoribosylpyrophosphate amidotransferase that catalyses first step of the '*de novo*' purine nucleotide biosynthetic pathway was repressed by downregulation of *ADE4*, *ADE1*, *ADE5,7* and *ADE13* genes. The ribosome biogenesis was also inhibited by suppression of *RPA'*s (*RPA190, RPA49, RPA43* and *RPA135*) as known RNA polymerase I largest subunits. Many of genes were declined about RNA processing that controlled by UTP genes. Besides, expression of the tRNAs is known to be repressed following a variety of stresses, including amino acid and nitrogen starvation, progression into stationary phase, defects in secretion, and DNA damage (Gasch, 2003). BA8 had a parallel downregulation maps that explained by Gasch *et al.*, (2000) as a repression of rDNA and ribosome protein genes, inhibition of tRNA synthesis is a general feature of the ESR.

In BA8, cholesterol and heme biosynthesises were also fairly reduced to prevent decrease of acetyl coA as highly used to produce for energy production. It was observed that boron resistant strain guide to various pathways to satisfy energy starvation. Because of that energy required metabolism and opponent metabolism that compete with energy pathways for same sources to use were repressed, when energy generating mechanisms were considerably upregulated.

Last of all, it was understood that evolutionary engineered BA8 has an important capability to tolerate boron rather than wild type. DNA microarray analysis demonstrates that upregulation and downregulation of lots of genes in BA8 would also further identified to have a better understanding of boron resistance mechanism of BA8. The results of transcriptomic analysis also searched out that BA8 up and downregulated genes similar to ESR system as a centre of known general stress response. In BA8, energy production pathways were highly activated and conserved to supply high amount of energy requirements, thus most of upregulated genes were related to energy metabolism. Furthermore, BA8 showed many resistance mechanisms mainly based on oxidative stress. Many genes involved in oxidative

stress resistance were also upregulated in BA8. Conversely BA8 seemed to have that it induced downregulation of many genes as associated to transcription and translation process with toxic effect of boron on Gcn2 that also called uncharged tRNA binding domain (HisRS). It might be a way to conserve energy that requires for production of ribosome.

5. FUTURE ASPECTS

The evolutionary engineering method is based on random mutagenesis and selection. Thus, it required a molecular analysis to determine the main factors conferring boron resistance in BA8. Boron related genes might be deleted with transformation process to understand how their absence can affect resistance mechanism. Actually the best way is creating whole genome library to elicit which genes are related to boron mechanism in BA8. Additional verification can be done by quantitative real time PCR. Moreover, whole genome sequence analysis can be carried out to detect DNA level changes in boron-resistant mutant BA8. Besides, proteomic analysis can be performed to better understand the underlying mechanisms. The better molecular characterization of boron-resistant strain might provide data for industrial applications with improved efficiency.

A. APPENDIX:

Table A.1: List of all up-regulated and down-regulated genes in boron resistant BA8 mutant. The genes which have fold change lower than 2 are not represented.

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
106,21	FMP43	23,34	PHO84
57,90	HSP12	15,07	ZRT1
54,23	HXK1	14,10	ARO3
46,42	STL1	5,75	ERG20
34,95	YNL194C	5,54	PDC5
34,63	FMP45	5,25	DBP2
33,35	YFL015C	5,08	YAR075W
32,60	TMA10	5,02	RPG1
32,03	BDH2	5,00	RSA4
27,94	DDR2	4,84	NSR1
27,70	SSA4	4,80	RIX7
27,33	ALD3	4,79	AI3
27,26	HSP26	4,68	KRE33
26,28	FMP16	4,46	EBP2
26,23	PGM2	4,41	CIC1
24,45	GPH1	4,41	REX4
23,71	YBR116C	4,36	BFR2
22,56	YCL046W	4,34	ADH4

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
22,47	CTT1	4,30	ARX1
22,41	YGR066C	4,22	NOG1
21,48	RTN2	4,22	RPC53
21,41	PRR2	4,20	EFG1
21,38	YCL041C	4,16	FPR4
21,18	HXT7	4,10	ESF2
20,86	PHM7	4,10	DHR2
19,86	GTO3	4,09	SDA1
19,60	TKL2	4,04	GRC3
18,26	RSB1	4,02	IPI1
17,96	YOR186W	3,97	DBP7
17,57	YFL012W-A	3,88	RRP9
17,48	YPR002C-A	3,86	TRM44
16,72	YOR314W-A	3,83	HMT1
16,71	SOL4	3,80	IPI3
16,12	YML099W-A	3,80	SUL1
16,01	HXT5	3,79	NOP4
15,97	HXT6	3,76	DBP9
15,77	YMR206W	3,68	YAR073W
15,72	CYC7	3,65	RRP8
15,55	YER067W	3,60	PNO1
14,75	YNR034W-A	3,60	NOG2
14,67	YLR162W	3,58	KRE29
14,63	ADY2	3,57	MRD1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
14,51	HSP78	3,55	NOP9
14,08	POT1	3,55	EHD3
14,03	BTN2	3,53	ATF2
13,83	YGR022C	3,49	RIO1
13,49	YCR013C	3,49	MRT4
12,88	YIL060W	3,47	RLP24
12,81	DCS2	3,44	HGH1
12,64	YDL023C	3,43	FCY2
12,27	YBR178W	3,41	SPB1
12,17	SLZ1	3,37	NSA2
12,04	APJ1	3,34	LCP5
11,80	YGP1	3,33	NOP2
11,73	YGR176W	3,33	RRB1
11,68	YDR374C	3,32	NRP1
11,40	SPG4	3,28	NOP53
11,09	ENA2	3,28	ALB1
11,08	XBP1	3,24	ECM16
11,01	YIL057C	3,23	SGD1
11,00	GLC3	3,22	UTP22
10,96	NQM1	3,20	UTP18
10,93	ATO2	3,19	AAH1
10,85	MBR1	3,18	DIP2
10,63	YDR034C-A	3,18	RPB5
10,55	YLR252W	3,17	NOC2

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
10,26	HSP42	3,17	YBL073W
10,24	PNS1	3,17	YML090W
10,18	YHL005C	3,16	UTP14
10,17	RTC3	3,15	TIR2
10,08	MSC1	3,14	UTP21
10,04	GAT4	3,12	PUF6
9,89	SUE1	3,11	RTT10
9,87	YFR017C	3,10	NMD3
9,83	SPS100	3,09	BMS1
9,78	YJL144W	3,09	MTR4
9,69	ALD4	3,08	SPO12
9,67	SPI1	3,07	PHM6
9,53	GAD1	3,07	SOF1
9,53	YNL195C	3,07	SSF1
9,49	ISF1	3,07	YIL091C
9,49	GAC1	3,06	DRS1
9,43	YNL143C	3,05	SHQ1
9,31	YGL052W	3,04	YCR016W
9,23	SGA1	3,04	URA7
9,22	OM14	3,02	RRP12
9,17	YOR345C	3,02	YMC2
8,95	SRX1	3,01	MAK5
8,94	PEX18	3,01	UTP13
8,83	YDR366C	2,97	DAD2

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
8,73	YGL109W	2,96	RIO2
8,66	MAM1	2,96	AAT1
8,60	NCE103	2,95	TSR2
8,50	GPM2	2,94	ENP1
8,48	GLK1	2,94	IMD4
8,36	CWP1	2,94	ECM1
8,06	YAL004W	2,90	YKL027W
7,97	YDR426C	2,88	RAS1
7,95	YBL108W	2,87	HCA4
7,92	YHR049C-A	2,87	NUG1
7,89	IRC15	2,87	TSR1
7,89	YDR535C	2,85	UTP4
7,88	GRE1	2,85	DBP8
7,85	SYM1	2,84	NOC3
7,85	YJR087W	2,83	RPF2
7,85	YPL142C	2,82	NOP7
7,74	YNL120C	2,82	PWP1
7,71	MTH1	2,81	TOD6
7,71	YLR312C	2,80	SAS10
7,70	YHR022C	2,80	NOC4
7,65	YOL079W	2,79	PUS1
7,65	YKR075C	2,78	RPA49
7,59	HOP2	2,77	YTM1
7,58	YGR069W	2,77	RRS1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
7,57	UIP4	2,77	RPA43
7,54	YKL091C	2,76	FOL1
7,51	STF1	2,76	EFG1
7,50	USV1	2,74	YNL313C
7,44	SDS24	2,72	BUD23
7,42	YMR245W	2,72	UTP23
7,37	GPG1	2,72	HIS1
7,36	YOR055W	2,71	PRP24
7,33	YLR053C	2,71	URA1
7,32	YBR285W	2,71	YMR193C-A
7,31	YLR149C	2,71	TRM1
7,28	Q0182	2,70	AIR1
7,25	MAL12	2,70	YVH1
7,18	YGL182C	2,68	RRP5
7,18	YMR082C	2,68	HAS1
7,16	YLR012C	2,68	ELP2
7,11	TPS2	2,68	UTP10
7,10	YGR035C	2,68	SRP72
7,08	TFS1	2,67	RRP36
7,04	GPD1	2,67	TRM11
7,02	YKL066W	2,67	NOP8
6,95	YKL107W	2,67	BCP1
6,94	EMI2	2,67	RNA14
6,90	MAL11	2,66	NAF1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
6,90	YJR079W	2,66	BUD22
6,88	MRK1	2,65	NOP14
6,83	PUT4	2,65	LSG1
6,78	MUM3	2,65	FYV7
6,75	REG2	2,65	PCS60
6,69	PRX1	2,64	FAF1
6,58	YOR029W	2,64	SAP185
6,54	COX5B	2,63	YIL096C
6,54	GSY2	2,63	TGS1
6,52	YPR044C	2,62	YCR087C-A
6,51	YLR311C	2,62	DBP10
6,51	GSY1	2,61	YBL081W
6,50	YDL228C	2,61	SSP1
6,49	AIM17	2,61	VTS1
6,48	YIL032C	2,60	SCD6
6,48	HSP82	2,59	HEM12
6,40	Q0010	2,58	TMA46
6,38	YMR090W	2,58	ERB1
6,36	IME4	2,57	YLR143W
6,35	FMP33	2,57	RPC82
6,34	YKL147C	2,57	PWP2
6,30	YNR014W	2,56	MPP10
6,29	MAL32	2,56	PUS7
6,25	YLR171W	2,56	YOR008W-B

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
6,22	YPL182C	2,56	RIX1
6,21	YJL163C	2,56	UTP8
6,17	YMR046W-A	2,56	NCL1
6,15	SIP18	2,55	TRM7
6,05	SSE2	2,55	TRM3
5,86	YMR326C	2,55	YDL151C
5,84	YGR164W	2,53	YLR413W
5,83	YDR491C	2,52	RSA1
5,83	YLR198C	2,52	SSN3
5,79	HSP104	2,51	CTP1
5,78	YTP1	2,51	FAA4
5,75	CYB2	2,51	SER2
5,73	YDR525W	2,50	RAI1
5,72	GIP2	2,50	GCD10
5,71	YNL179C	2,50	PRM7
5,69	YPL197C	2,50	RPA135
5,68	GND2	2,49	YNL022C
5,68	YDR278C	2,49	SLX9
5,67	YKR047W	2,47	RGS2
5,58	YKL036C	2,47	IMP3
5,55	YDL114W	2,47	YHM2
5,53	SFC1	2,46	MAK21
5,52	YDR379C-A	2,46	IMP4
5,52	Q0017	2,46	FAL1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
5,47	YLR062C	2,45	OTU2
5,46	SSA1	2,45	CLN2
5,46	YKL102C	2,45	BRX1
5,44	YKL151C	2,44	DAS2
5,42	PTR2	2,43	NUC1
5,40	AGX1	2,43	ADE4
5,38	SDP1	2,43	UTP5
5,38	YGL088W	2,42	DSE4
5,32	NDE2	2,41	PPR1
5,30	YBR099C	2,41	RKM4
5,29	YNL200C	2,41	YBR271W
5,28	MTL1	2,41	SUI2
5,27	YER053C-A	2,40	RSC58
5,27	YJR096W	2,40	MAK11
5,26	YIL100W	2,40	DBP3
5,20	YEL045C	2,39	PPH3
5,19	CIN5	2,39	MCH5
5,17	YML034C-A	2,38	DUS1
5,16	YJR115W	2,37	ESF1
5,13	OM45	2,37	NIP7
5,11	ECM8	2,37	YML082W
5,11	YLR076C	2,37	CBF5
5,10	TPS1	2,36	RFC4
5,07	YDR114C	2,36	PPT1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
5,07	YML089C	2,36	YDL063C
5,06	YGL034C	2,36	UTP9
5,02	YLR030W	2,36	RFU1
5,00	YOL150C	2,36	YBR141C
5,00	YLR302C	2,36	ENP2
4,99	YER079W	2,35	NSA1
4,99	ROM1	2,35	RKI1
4,95	YHL008C	2,34	ROK1
4,93	FDH1	2,34	INO2
4,93	YER121W	2,34	YLR363W-A
4,92	YKL153W	2,34	NOP13
4,91	YHR145C	2,34	RRP15
4,90	YPR099C	2,34	NOB1
4,89	YIL029C	2,33	TRM13
4,89	YBR124W	2,33	POL5
4,89	YDR509W	2,32	MTC3
4,88	YIL059C	2,32	SSF2
4,88	YLR296W	2,31	MTD1
4,87	YDR521W	2,30	RRP1
4,86	YNL028W	2,30	RCL1
4,83	YOR041C	2,30	YLH47
4,83	YHR095W	2,30	NTR2
4,80	UTR5	2,29	NOP15
4,77	SAP4	2,29	YHL039W

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
4,76	YNL105W	2,29	WRS1
4,75	YPR127W	2,29	CNS1
4,74	HOR2	2,29	URB2
4,73	YKR073C	2,29	YMR010W
4,72	AQY1	2,28	TRM8
4,72	YDR455C	2,28	FCF2
4,71	TPK1	2,27	PUS4
4,70	GRE3	2,27	SUT2
4,70	YGR182C	2,26	RRT14
4,69	AGP2	2,26	FAP7
4,67	MUC1	2,26	RPC17
4,64	YDL240C-A	2,26	YGR054W
4,63	GCY1	2,25	KSS1
4,62	FYV12	2,25	AFG2
4,62	YAL042C-A	2,25	TRM10
4,62	YCT1	2,25	TPA1
4,62	YDR018C	2,25	YLR235C
4,62	YFL019C	2,25	NHA1
4,61	IKS1	2,24	YCR051W
4,61	RIM4	2,24	DUS3
4,60	YGL042C	2,24	ADE1
4,59	ARO9	2,24	SKG6
4,57	OSW1	2,24	VPS74
4,57	YGR018C	2,24	PPX1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
4,57	GUT2	2,23	NAN1
4,56	RTS3	2,23	GEP3
4,55	FIT1	2,23	ATO3
4,55	YIL054W	2,22	KRI1
4,54	TMA17	2,22	URB1
4,53	YLL044W	2,22	SWI4
4,52	YGL081W	2,21	ZUO1
4,52	PRM2	2,20	GCN3
4,52	YLL037W	2,20	MKC7
4,51	YCR097W-A	2,20	SMI1
4,50	YDR112W	2,20	REI1
4,49	PRM10	2,20	TIF5
4,49	YER188W	2,19	UBP8
4,49	YCR041W	2,19	TRM2
4,48	YRO2	2,19	TSR4
4,48	YJR071W	2,19	CGR1
4,48	YSW1	2,18	ACA1
4,47	YBL100C	2,18	LYS4
4,47	YDL034W	2,18	HBS1
4,46	YDR230W	2,18	SRO9
4,45	CUR1	2,18	NCS2
4,45	BSC5	2,18	SHM2
4,43	GSP2	2,17	IFH1
4,43	YGR109W-A	2,17	MSH1

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
4,42	YKL097C	2,17	YPL277C
4,42	ADR1	2,17	NOP16
4,40	YJL142C	2,17	SVF1
4,40	COX1	2,16	NOP12
4,40	YLR184W	2,16	RNT1
4,39	YLL030C	2,16	UTR2
4,39	ECM11	2,16	PRP28
4,39	YJR023C	2,16	MET22
4,37	PIG2	2,16	RET1
4,37	YLR365W	2,15	POR2
4,34	TDH1	2,15	UBP10
4,31	ZPR1	2,15	FUR1
4,30	GAL7	2,14	UTP11
4,29	YHR140W	2,14	FRS1
4,26	YML007C-A	2,14	FOB1
4,24	YLR124W	2,14	YDR161W
4,24	YGL041C	2,13	LIA1
4,24	YPR064W	2,13	UBP12
4,21	YKL133C	2,13	OGG1
4,20	YBR051W	2,13	PRP19
4,17	HXT15	2,12	ALT2
4,17	YDL062W	2,12	PCL1
4,15	YOR082C	2,12	YOR051C
4,15	MDH2	2,12	TIF35

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
4,14	SIS1	2,12	YLR126C
4,13	FDH1	2,11	NOP1
4,10	CRC1	2,11	RIF1
4,09	CLD1	2,11	KEL3
4,07	DIA3	2,11	UTP7
4,07	YLR177W	2,11	MMT1
4,05	YJL064W	2,11	UTP20
4,05	AAC1	2,11	PRM7
4,05	YGL230C	2,11	RPA190
4,03	YLR345W	2,10	TRF5
4,03	YBL109W	2,10	ATC1
4,03	GAL2	2,10	RLI1
4,02	YCL007C	2,09	CYB5
4,01	UBI4	2,09	NOP58
4,00	ATG14	2,09	TRL1
3,99	YER091C-A	2,08	LTV1
3,98	YOR139C	2,08	FAP1
3,94	YIL025C	2,08	FIN1
3,92	SED1	2,08	YJR124C
3,92	DIT2	2,08	YLR243W
3,91	PDR15	2,08	SQT1
3,91	HSP30	2,08	EKI1
3,91	YGR067C	2,07	ADE5,7
3,90	LEE1	2,07	LEU9

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,87	YPR170C	2,06	IMD2
3,85	STB2	2,06	RPO31
3,85	UGP1	2,06	FUN12
3,84	YCL042W	2,06	PLB2
3,83	YGL036W	2,06	YNL095C
3,83	XKS1	2,06	EMP70
3,82	AIM26	2,06	YOL014W
3,82	YDR250C	2,05	CDC8
3,82	ICL1	2,05	LHP1
3,81	RRT6	2,05	HO
3,80	JEN1	2,04	URK1
3,80	YPR012W	2,04	NMA111
3,80	YJL185C	2,04	GEA2
3,80	YDL009C	2,03	SSB1
3,79	YER039C-A	2,03	FUI1
3,78	VHS1	2,03	IRC7
3,78	YBL077W	2,03	TMA16
3,78	YER097W	2,03	YLR460C
3,78	FMP23	2,02	FAR1
3,78	YLR415C	2,02	TAF7
3,77	PHM8	2,02	RLP7
3,76	ABM1	2,02	RPC34
3,75	GRE2	2,02	RMT2
3,74	YLR346C	2,02	TRM82

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,73	PDC6	2,02	SFG1
3,72	ECM12	2,01	ADE13
3,71	ENA1	2,01	NNF2
3,71	YAL034C-B	2,01	RPS8B
3,69	YNL043C	2,00	YHP1
3,69	FES1	2,00	CDC3
3,68	YLR269C	2,00	HFI1
3,68	SPR3		
3,67	ULA1		
3,67	GAL10		
3,66	YNL305C		
3,65	GLG1		
3,64	PRM1		
3,62	YLR111W		
3,61	YKL225W		
3,61	STF2		
3,60	BSC4		
3,59	YKR015C		
3,59	YML131W		
3,59	MEK1		
3,59	YGR131W		
3,58	IDP2		
3,58	MLS1		
3,57	PNC1		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,56	YOL047C		
3,54	YGR236C		
3,53	YAL069W		
3,53	YDR360W		
3,52	THI4		
3,52	YHR097C		
3,51	INO1		
3,51	YMR290W-A		
3,50	YJR114W		
3,49	YNL276C		
3,49	AIM19		
3,49	YIL055C		
3,49	YOR121C		
3,49	COB		
3,49	RRT8		
3,49	GPX1		
3,48	YPL247C		
3,48	YBR089W		
3,47	YPT53		
3,47	YMR194C-A		
3,47	PIC2		
3,46	VPS73		
3,46	YCR022C		
3,46	YGL217C		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,46	RRT5		
3,45	PRY1		
3,45	YIR016W		
3,45	YOL106W		
3,45	FDH1		
3,42	YFL063W		
3,42	COQ9		
3,41	YMR262W		
3,41	YJL107C		
3,41	YLR379W		
3,40	SMK1		
3,40	YJR120W		
3,40	YML053C		
3,39	RTC2		
3,38	RNP1		
3,36	NTH1		
3,35	CIT1		
3,35	YGL218W		
3,35	YAR068W		
3,34	YOL134C		
3,33	COX2		
3,33	YLR108C		
3,33	YNR068C		
3,32	COX7		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,32	YOR152C		
3,32	COX3		
3,32	GRX1		
3,32	CTA1		
3,32	HBT1		
3,31	YGL258W-A		
3,31	PAI3		
3,31	OPI10		
3,31	YAL056C-A		
3,29	YPL062W		
3,29	ECM4		
3,28	SOL1		
3,27	YOL118C		
3,27	YAR068W		
3,25	NDI1		
3,24	YPL185W		
3,24	YBR085C-A		
3,23	AMS1		
3,23	YDR048C		
3,23	YDR034W-B		
3,23	FMP40		
3,23	YOL024W		
3,23	YOR292C		
3,22	YHR033W		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,22	YIR014W		
3,21	CAR2		
3,20	ICS3		
3,20	GRX2		
3,20	MAL31		
3,18	PHO89		
3,18	Q0297		
3,18	YDR526C		
3,18	ETR1		
3,18	YKL169C		
3,17	HPF1		
3,17	YGR053C		
3,16	CRF1		
3,16	HVG1		
3,16	YGL117W		
3,15	MGA1		
3,15	HOR7		
3,15	NCE102		
3,13	YJL188C		
3,13	YSC84		
3,13	CAT8		
3,12	YPR077C		
3,12	YOR289W		
3,12	YJL156W-A		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,11	YMR196W		
3,11	GIS1		
3,11	YGL138C		
3,10	JIP4		
3,09	CAR1		
3,09	YDL026W		
3,09	YMR291W		
3,08	TSA2		
3,07	PRM8		
3,07	YNR001W-A		
3,06	YML012C-A		
3,06	YGL015C		
3,06	YBR284W		
3,05	CSR2		
3,04	MST27		
3,03	YGL102C		
3,02	YLR159W		
3,02	HUG1		
3,02	POX1		
3,02	YCR064C		
3,01	GDB1		
3,01	YDL162C		
3,01	TES1		
3,00	YDL096C		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
3,00	YCR085W		
3,00	TBS1		
3,00	YLR338W		
3,00	UBC8		
2,99	YDR271C		
2,99	ARG82		
2,98	YBR053C		
2,98	YMR118C		
2,98	GTO1		
2,98	SPO13		
2,98	EDC2		
2,96	PRM6		
2,96	YNL228W		
2,96	YAR053W		
2,94	YLR161W		
2,94	YOR062C		
2,92	ASG7		
2,92	PBI2		
2,92	YGR205W		
2,92	BAP3		
2,91	YPR015C		
2,91	DTR1		
2,91	YJR020W		
2,91	YPL251W		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,91	GSC2		
2,90	ICS2		
2,90	ADD37		
2,90	SDH1		
2,89	YLR164W		
2,89	TPS3		
2,89	YGL007W		
2,89	RNY1		
2,89	AVO2		
2,88	YPS5		
2,88	MRP10		
2,88	LSP1		
2,88	YJL182C		
2,87	YEL008W		
2,87	SPR28		
2,87	GLG2		
2,86	YLR156W		
2,86	YJL120W		
2,85	YNL266W		
2,85	STE18		
2,85	OYE3		
2,85	ATH1		
2,84	YKL136W		
2,84	YLR230W		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,83	YBL029C-A		
2,83	ATP18		
2,83	RPN4		
2,83	ATP6		
2,83	SIP2		
2,82	UGX2		
2,82	NDL1		
2,81	FMP46		
2,81	YOR378W		
2,81	YIL169C		
2,81	YPL014W		
2,81	YOR225W		
2,81	YDR417C		
2,80	JID1		
2,80	MRP8		
2,79	VID30		
2,79	COX14		
2,78	AGP1		
2,78	ATP15		
2,78	YPS6		
2,78	DOA4		
2,78	QCR10		
2,76	MCR1		
2,76	YER066C-A		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,76	PTK2		
2,76	MDJ1		
2,76	YML047W-A		
2,75	RCR2		
2,75	YIL077C		
2,75	YDR209C		
2,73	YCR018C-A		
2,73	YER084W		
2,72	YGR228W		
2,72	YJL195C		
2,72	SNA2		
2,72	ACH1		
2,71	PCD1		
2,71	YMR173W-A		
2,71	MATALPHA1		
2,71	ZRG8		
2,71	YIL058W		
2,70	YAK1		
2,70	YDL041W		
2,69	MUP3		
2,69	YKR040C		
2,69	MBF1		
2,69	YET2		
2,68	YMR141C		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,68	YGR107W		
2,68	PDE1		
2,67	PCK1		
2,67	LEU5		
2,66	FRT2		
2,66	LST8		
2,66	YIL102C		
2,66	YNL285W		
2,66	YCR050C		
2,66	YGR201C		
2,66	YJL199C		
2,65	YOR364W		
2,65	YCR038W-A		
2,64	SGF11		
2,64	YPR130C		
2,64	MET32		
2,63	SPS19		
2,63	YJR012C		
2,63	TRX2		
2,63	MET28		
2,62	YGR293C		
2,62	GLO1		
2,62	UBA3		
2,62	YMR052C-A		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,62	YBL044W		
2,61	YBR277C		
2,61	YJL022W		
2,61	ICY1		
2,61	HLR1		
2,60	YAP1801		
2,60	VHS3		
2,60	FCY22		
2,59	YPT35		
2,59	PET18		
2,58	GPT2		
2,58	YDR136C		
2,58	JIP4		
2,58	AIM18		
2,57	ICY2		
2,57	YLR408C		
2,57	YAL045C		
2,57	YLR101C		
2,56	URA10		
2,55	YOR214C		
2,55	SOH1		
2,55	REC102		
2,54	YKL165C-A		
2,54	YJL015C		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,54	HAL1		
2,54	YGR130C		
2,53	TVP15		
2,53	YHL037C		
2,53	YBR209W		
2,52	YNL109W		
2,52	MPH3		
2,52	SHC1		
2,52	QCR7		
2,51	NCE101		
2,51	FUN19		
2,51	CTR3		
2,50	KAR9		
2,50	HXT16		
2,50	CAT2		
2,49	YLR349W		
2,49	DDR48		
2,49	YKL177W		
2,49	YPL056C		
2,49	SDH3		
2,49	ACP1		
2,48	HSP33		
2,48	YKL053W		
2,48	YKL111C		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,47	FOX2		
2,47	DCS1		
2,46	NTH2		
2,46	YHR217C		
2,46	GGA1		
2,46	MVB12		
2,46	YBL048W		
2,45	YDL025C		
2,45	SFT1		
2,45	YDL199C		
2,45	BBC1		
2,45	YDL206W		
2,45	YJL175W		
2,44	YFL040W		
2,44	YCR061W		
2,44	YGR050C		
2,44	YJL067W		
2,44	MXR2		
2,43	MYO3		
2,42	ZPS1		
2,42	XYL2		
2,42	OPT1		
2,42	YML122C		
2,42	YLR446W		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,42	OPT2		
2,42	COX20		
2,41	RCN2		
2,41	YDR053W		
2,40	ZTA1		
2,40	YNL150W		
2,40	YBR103C-A		
2,40	YDR327W		
2,40	YDR431W		
2,40	TIM11		
2,40	SPO23		
2,39	HUR1		
2,39	CSI1		
2,38	TC21003		
2,38	SKN1		
2,38	YDR543C		
2,38	YLR202C		
2,38	ASI3		
2,37	QCR9		
2,37	PEX22		
2,37	YJL070C		
2,37	DFG10		
2,37	BOP2		
2,36	CIS1		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,36	YOL162W		
2,36	DAS1		
2,36	LAP4		
2,35	BNS1		
2,35	CUP1-1		
2,35	YKL030W		
2,35	ECM19		
2,35	AUA1		
2,35	YBR013C		
2,34	FUN14		
2,34	OLI1		
2,34	EMP46		
2,34	YPR059C		
2,34	PLM2		
2,34	UGA2		
2,33	UBX3		
2,33	YGR269W		
2,33	SRT1		
2,33	ROX1		
2,33	YPC1		
2,33	VEL1		
2,33	YIG1		
2,33	FUS1		
2,32	SRL1		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,32	YJR008W		
2,32	YMR151W		
2,32	CCP1		
2,32	YLR152C		
2,32	SNT309		
2,31	CUP1-2		
2,31	UGA1		
2,31	YNL198C		
2,31	PKP1		
2,31	CCW12		
2,30	GIS3		
2,30	AGA2		
2,30	HMRA2		
2,30	YDL071C		
2,29	RSM27		
2,29	SPO20		
2,29	YGR122C-A		
2,29	SIP4		
2,28	UBS1		
2,28	YBR113W		
2,28	LCB3		
2,28	YKL171W		
2,28	MND1		
2,27	YAT2		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,27	ANS1		
2,26	MAM3		
2,26	YOR223W		
2,26	MER1		
2,26	YOR169C		
2,26	YGR259C		
2,25	SHE9		
2,25	ENA5		
2,25	YOR135C		
2,24	RFS1		
2,24	HSE1		
2,24	YHL044W		
2,24	STR3		
2,24	YBL112C		
2,23	SMT3		
2,23	YPL238C		
2,23	PYK2		
2,23	YCL057C-A		
2,23	AAD3		
2,23	YIL028W		
2,23	PAD1		
2,23	HSP32		
2,22	YOR331C		
2,22	HFD1		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,22	SNA4		
2,22	YPL272C		
2,22	YDL185C-A		
2,21	YMR085W		
2,21	REC114		
2,21	YML101C-A		
2,21	NAT4		
2,20	MTQ1		
2,20	YCL056C		
2,20	IDP3		
2,20	FBP1		
2,20	YJL086C		
2,20	SOD2		
2,20	ECL1		
2,20	YGL239C		
2,20	MRP17		
2,20	YNL011C		
2,19	PFK26		
2,19	YGL188C		
2,19	YLR334C		
2,19	AIM7		
2,19	HUL4		
2,19	GTT1		
2,19	YLR255C		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,19	YOL085C		
2,19	HER1		
2,19	DAL80		
2,19	YLR402W		
2,19	VPS71		
2,18	YJR018W		
2,18	SPR6		
2,18	PIR3		
2,18	TIM18		
2,18	RRI2		
2,18	FIG1		
2,18	YJL132W		
2,18	RNR3		
2,18	DAN4		
2,18	RMD5		
2,18	OXR1		
2,17	YBR012C		
2,17	RMR1		
2,17	UGO1		
2,17	YNK1		
2,17	YJR085C		
2,17	YGR242W		
2,17	FYV10		
2,17	DSN1		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,16	BNA3		
2,15	GAS4		
2,15	PKH1		
2,15	YJR162C		
2,15	SNF11		
2,15	VBA3		
2,15	YLR112W		
2,15	ADH7		
2,15	DNL4		
2,14	ATP19		
2,14	YAL018C		
2,14	ACN9		
2,14	YML084W		
2,14	HST4		
2,13	MRP20		
2,13	YOR318C		
2,13	LSM8		
2,13	YEL057C		
2,13	MOH1		
2,12	SAM37		
2,12	MED7		
2,12	YNR077C		
2,12	MPH2		
2,12	SCS3		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,12	COQ4		
2,12	PGS1		
2,11	YFL067W		
2,11	NAB3		
2,11	TUL1		
2,11	YEL074W		
2,11	VID24		
2,11	YFR045W		
2,11	YJL147C		
2,10	YMR181C		
2,10	YNL034W		
2,10	BI3		
2,10	YLR366W		
2,10	DIG2		
2,10	EST3		
2,10	SNO4		
2,10	SRC1		
2,10	YGL072C		
2,09	YPR053C		
2,09	CRD1		
2,09	YLR218C		
2,09	YAP1802		
2,09	AIM41		
2,09	ADY3		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,09	YDR532C		
2,09	YIR007W		
2,08	DMC1		
2,08	ADA2		
2,08	YCR025C		
2,08	YMR087W		
2,08	YGR127W		
2,08	ATG8		
2,08	TRR2		
2,08	YDL133W		
2,07	EMI5		
2,07	LPE10		
2,07	IML2		
2,07	PES4		
2,07	VMR1		
2,07	YMR244C-A		
2,07	AFR1		
2,07	COX13		
2,06	YPR078C		
2,06	YEF1		
2,06	RDS3		
2,06	GAL4		
2,06	YFL054C		
2,06	YOR235W		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,05	TAX4		
2,05	TPK2		
2,05	YPR050C		
2,05	YNL146W		
2,05	YFR035C		
2,04	YNL115C		
2,04	RRG10		
2,04	SEF1		
2,04	YLR290C		
2,04	DAD4		
2,04	YKL031W		
2,03	YPL025C		
2,03	GTT3		
2,03	YGR210C		
2,03	DUR3		
2,03	YCR076C		
2,03	YBR241C		
2,03	SOP4		
2,03	SDH4		
2,03	Ara.01		
2,03	BDH1		
2,03	SNC1		
2,02	YBR139W		
2,02	AVT6		

Fold Change (Up-regulation)	Gene Symbol	Fold Change (Down-regulation)	Gene Symbol
2,02	FMP21		
2,02	YMR320W		
2,02	AI5_ALPHA		
2,01	MPM1		
2,01	PTC6		
2,01	YCL001W-B		
2,01	PIN3		
2,01	COS12		
2,01	PRC1		
2,01	POC4		
2,01	YNL296W		
2,01	YDR042C		
2,01	PPE1		
2,00	VHR1		
2,00	YOR019W		
2,00	YBR056W		
2,00	LDB16		

6. REFERENCES

Ahmed, I., A. Yokota, et al. (2007). "A novel highly boron tolerant bacterium, Bacillus boroniphilus sp. nov., isolated from soil, that requires boron for its growth." *Extremophiles* **11**(2): 217-224.

Alberts, B. Johnson, A., Lewis, J., Raff, M., Bray, D., Hopkin, K., Roberts, K., Walter, P. (1998). Essential Cell Biology. An Introduction to the Molecular Biology of the Cell, Garland Publishing.

Alberts, B., Johnson A., Lewis J., Raff M., Roberts K., Walter P. (2002). Molecular Biology of The Cell, 4th edition. New York, Garland Science.

Anderson, G. R. and J. V. Jordan (1961). "Boron: A Non-Essential Growth Factor for Azotobacter Chroococcum." *Soil Science* 92(2): 113.

Bailey, J.E., Sburlati, A., Hatzimanikatis, V., Lee K., Renner, W.A., Tsai, P.S. (1996). Inverse Metabolic Engineering: a Strategy for Directed Genetic Engineering of Useful Phenotypes, *Biotechnology and Bioengineering*, 52, 109-121.

Bonilla, L., Gonzalez, M.G., Mateo, P., (1990). Boron Requirement in Cyanobacterial Its Possible Role in the Early Evolution of Photosynthetic Organisms, *Plant Physiol.*, 94, 1554-1560.

Çakar, Z.P., Alkım, C., Turanlı, B., Tokman, N., Akman, S., Sarıkaya, M., Tamerler, C., Benbadise, L., Françoise, J. M., (2009). Isolation of Cobalt Hyper-resistant Mutants of Saccharomyces cerevisiae by in vivo Evolutionary Engineering Approach, *Journal of Biotechnology*, 143, 130–138.

Çakar, Z.P., Şeker, U.Ö.Ş., Tamerler, C., Sonderegger, M., Sauer, U., (2005). Evolutionary Engineering of Multiple-stress Resistant *Saccharomyces cerevisiae*, *FEMS Yeast Res.*, 5, 569-578.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S., Young, R.A. (2001). Remodeling of yeast genome expression in response to environmental changes. Mol Biol Cell. 12(2):323-37.

Coulthurst, S.J., Whitehead, N.A., Welch, M., Salmond, G.P.C. (2002). Can boron get bacteria talking? *Trends Biochem. Sci.* 27, 217–219.

Dell, B., and Huang, L. (1997) Physiological response of plants to low boron. Plant Soil 193: 103–120.

Dorak, M. T. (2006). *Real-time PCR*. Taylor Francis, London.

Egilmez, N.K., Jazwinski, S.M. (1989). Evidence for the involvement of a cytoplasmic factor in the aging of the yeast Saccharomyces cerevisiae. J. Bacteriol. vol. 171 no. 1 37-42

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. PNAS vol. 95 no. 25 14863-14868.

Eitinger, T., Mandrand-Berthelot, M. A. (2000). Nickel transport systems in microorganisms. *Arch Microbiol, 173*(1), 1-9.

Elion, E.A. (2000). Pheromone response, mating and cell biology.Curr Opin Microbiol 3, 573–581.

Eyster, C. (1958). The mıcro-element nutrıtıon of *Nostoc muscorum*. The ohıo journal of scıence 58(1): 25

Federoff, H. J., Eccleshall, T. R., Marmur, J. (1983). Carbon catabolite repression of maltase synthesis in *Saccharomyces carlsbergensis*. J. Bacteriol. 156:301–307.

Francois, J., Parrou, J. L. (2001). Reserve carbohydrates metabolism in the yeast Saccharomyces cerevisiae. *FEMS Microbiol Rev*, *25*(1), 125-145.

Garcia-Gonzalez, M., Mateo, P., Bonilla, I., (1988). Boron Protection for O_2 diffusion in Heterocysts of Anabaena sp. PCC 71191, *Plant Physiol.*, 87, 785-789.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., Brown, P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell, 11*(12), 4241-4257.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., Brown, P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell 11:4241–4257.

Gasch, A.P., Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. Funct. Integr. Genomics 2(4-5):181-192.

Geoffrey, M.C. (2000). The Cell: A Molecular Approach, 2nd edition. Sinauer Associates,Inc.

Gerloff, G., C. (1968). The Comparative Boron Nutrition of Several Green and Blue-Green Algae. Physiologia Plantarum,Volume 21, Issue 2, 369–377.

Gill, R. T. (2003). Enabling İnverse Metabolic Engineering through Genomics, *Current Opinion in Biotechnology*, 14, 484–490.

Goldbach, H. E., Q. Yu, et al. (2001). "Rapid response reactions of roots to boron deprivation. " *Journal of Plant Nutrition and Soil Science* 164(2): 173-181.

González-Párraga, P., Sánchez-Fresneda, R., Martínez-Esparza, M., Argüelles, J.-C. (2008). Stress responses in yeasts: what rules apply? *Arch Microbiol, 189*(4), 293-296.

Iwai, H., N. Masaoka, et al. (2002). "A pectin glucuronyltransferase gene is essential for intercellular attachment in the plant meristem." *Proceedings of the National Academy of Sciences of the United States of America* 99(25): 16319.

Jaafar, L., Zueco, J.(2004).Characterization of a glycosylphosphatidylinositolbound cell-wall protein (GPI-CWP) in Yarrowia lipolytica, *Microbiology*, 150, 53–60

Jennings, M.L., Howren, T.R., Cui, J., Winters, M., Hannigan, R., (2007). Transport and regulatory characteristics of the yeast bicarbonate transporter homolog Bor1p. Am J Physiol Cell Physiol 293: 468–476.

Joseph-Strauss, D., Zenvirth, D., Simchen, G., Barkai, N. (2007).Spore-germination in *Saccharomyces cerevisiae;* global gene expression patterns and cell cycle landmarks. *Genome Biol.* 8*:* R241.

Kanazawa, S., Driscoll, M., Struhl, K., (1988). ATR1, a *Saccharomyces cerevisiae* gene encoding a transmembrane protein required for aminotriazole resistance. Mol Cell Biol 8: 664-673.

Kaya, **A., Karakaya**, **H.C., Fomenko**, **D.E., Gladyshev**, **V.N., Koç, A.,** (2009). Identification of a Novel System for Boron Transport: Atr1 is a Main Boron Exporter in Yeast, *Mol Cell Biol.*, 29, 3665-74.

Kazanskii, A.G., Mell, H., Terukov, E. I., Forsh, P.A. (2002). Effect of boron dopant on the photoconductivity of microcrystalline hydrogenated silicon films, *Semiconductors*, 36, 41-44.

Lei, J., Zhao, X., Ge, X., Bai, F., (2007). Ethanol tolerance and the variation of plasma membrane composition of yeast floc populations with different size distribution. J Biotechnol 131: 270–275.

Lewin, J.C., (1966). Physiological Studies of the Boron Requirement of the Diatom, *Cylindrotheca fusiformis*, The *Journal of Experimental Botany*,17, 473-479.

Lewis, J. G., Learmonth, R. P., Watson, K. (1995). Induction of heat, freezing and salt tolerance by heat and salt shock in Saccharomyces cerevisiae. *Microbiology, 141 (Pt 3)*, 687-694.

Lewis, J.G., Learmonth, R.P., Attfield, P.V., Watson, K. (1997). Stress cotolarance and trehalose content in baking strains of *Saccharomyces cerevisiae*, Journal of Industrial Microbiology Biotechnology, 18, 30-36.

Lodish., Harvey., Arnold, B., Zipursky, S.L., Matsudaira, P., Baltimore, D., Darnell, J.E., New York: W H Freeman & Co; (1999). Molecular Cell Biology 4th Edition,
Available:http://www.ncbi.nlm.nih.gov/books/bv.fcgi?call=bv.View..ShowTOC&rid=mcb.TOC

Lukaszwski, K., Bonilla, I., Blevins, D. (2004). Why Boron? *Plant Physiology and Biochemistry*, 42, 907–912.

Madigan, M.T., Martinko, J.M., Parker, J. (2003). Brock Biology of Microorganisms, 10th Edition. Prentice Hall. USA.

Miwa, K., J. Takano, et al. (2007). "Plants tolerant of high boron levels." *Science* 318(5855): 1417.

Moseley, J. B., and Goode, B. L., (2006). "The yeast actin cytoskeleton: from cellular function to biochemical mechanism," *Microbiology and Molecular Biology Reviews*, vol. 70, no. 3, pp. 605–645.

Navarro-Aviño J. P., Prasad R., Miralles V. J., Benito R. M., Serrano R. (1999). A proposal for nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterization of the stress-inducible ALD2 and ALD3 genes. Yeast 15: 829–842.

Nozawa, A., Miwa, K., Kobayashi, M., Fujiwara, T. (2006). Isolation of Arabidopsis thaliana cDNAs that Confer Yeast Boric Acid Tolerance, *Biosci. Biotechnol. Biochem*., 70, 1724-1730.

Nozawa, A., Takano, J., Kobayashi, M., Nicolaus von Wir´en, Fujiwara.T., (2006). Roles of BOR1, DUR3, and FPS1in boron transport and tolerance in Saccharomyces cerevisiae FEMS Microbiol Lett 262 216–222.

Ogawa, Y., A, Nitta., Uchiyama, H., Imamura, T., Shimoi, H., Ito, K. (2000). Tolerance mechanism of the ethanol-tolerant mutant of sake yeast. J. Biosci Bioeng 90:334-45.

Ohnishi, G., Endo, K., Doi, A., Fujita, A., Daigaku, Y., Nunoshiba, T. (2004). Spontaneous mutagenesis in haploid and diploid Saccharomyces cerevisiae. Biochem Biophys Res Commun 325, 928 -933.

O'Neill, M. A., T. Ishii, et al. (2004). "Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide." *Annu. Rev. Plant Biol.* 55: 109-139.

Paquin, C., and Adams, J. (1983). Frequency of fixation of adaptive mutations is higher in evolving diploid than haploid yeast populations. Nature 302, 495–500.

Park, M., Q. Li, et al. (2004). "NaBC1 is a ubiquitous electrogenic Na+-coupled borate transporter essential for cellular boron homeostasis and cell growth and proliferation." *Molecular cell* 16(3): 331-341.

Perez-Castro, R., Kasai, K., Gainza-Cortes, F., Ruiz-Lara, S., Casaretto, A. J., Pena-Cortes, H., Tapia, J., Fujiwara, T., Gonzalez, E., (2012). VvBOR1, the Grapevine Ortholog of AtBOR1, Encodes an Efflux Boron Transporter That is Differentially Expressed Throughout Reproductive Development of Vitis vinifera L. Plant Cell Physiol. 53(2): 485–494

Perrot, V. (1994). Experimental approaches to the evolution of life cycles. Lect Math Life Sci 25, 121–134.

Petri, R., and Schmidt-Dannert, C. (2004). Dealing with Complexity: Evolutionary Engineering and Genome Shuffling, *Current Opinion in Biotechnology*, 15, 298–304.

Prutting, S. M., and Cerveny, J. D., (1998). "Boric acid vaginal suppositories: a brief review," *Infectious Diseases in Obstetrics and Gynecology*, vol. 6, no. 4, pp. 191–194.

Reed, R.H., Chudek, J.A., Foster, R. and Gadd, G.M., (1987). Osmotic significance of glycerol accumulation in exponentially growing yeasts. *Appl. Environ. Microbiol.* 53, 2119–2123.

Roth, R, Halvorson, H.O. (1969). Sporulation of yeast harvested during logarithmic growth. J Bacteriol 98:831-832.

Rowe, R. I. and C. D. Eckhert (1999). "Boron is required for zebrafish embryogenesis." *Journal of experimental biology* 202(12): 1649.

Ruepp, A., Zollner, A., Maier, D., Albermann, K., Hani, J., Mokrejs, M., Tetko, I., Guldener, U., Mannhaupt, G., Munsterkotter, M., and Mewes, H. (2004). The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Research, 32:5539-5545.

Sá-Correia, I., Dos Santos, S.C., Teixeira, M.C., Cabrito. T.R., Mira, N.P. (2008). Drug:H^+ antiporters in chemical stress response in yeast. Trends Microbiol 17: 22-31.

Schmidt, M., Bowers, B., Varma, A., Roh, D. H., Cabib, E., (2002). "In budding yeast, contraction of the actomyosin ring and formation of the primary septum at cytokinesis depend on each other " *Journal of Cell Science*, vol. 115, no. 2, pp. 293– 302.

Schmidt, M., Schaumberg, J.Z., Steen, C.M., Boyer, M.P., (2010). Boric Acid Disturbs CellWall Synthesis in Saccharomyces cerevisiae. International Journal of Microbiology Volume 2010, Article ID 930465, 10 pages

Schmidt, M., Varma, A., Drgon, T., Bowers, B., Cabib, E., (2003). "Septins, under Cla4p regulation, and the chitin ring are required for neck integrity in budding yeast," *Molecular Biology of the Cell*, vol. 14, no. 5, pp. 2128–2141.

Schmittgen, T.D., Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-1108.

Scott, M.P., Matsudaira, P., Lodish, H., Darnell, J., Zipursky, L., Kaiser, C.A., Berk, A., Krieger, M. (2004). *Molecular Cell Biology, Fifth Edition*. WH Freeman and Col, NY. ISBN0-7167-4366-3.

Sherman, F. (1998). An Introduction to the Genetics and Molecular Biology of the Yeast *Saccharomyces cerevisiae*. Modified from: F. Sherman. 1997. Yeast genetics. The Encyclopedia of Molecular Biology and Molecular Medicine. 6:302-325. Edited by R. A. Meyers, VCH Pub., Weinheim, Germany.

Smits, G.J., Brul, S. (2005). Stress tolerance in fungi to kill a spoilage yeast. *Curr Opin Biotechnol, 16*(2), 225-230.

Snustad, **P.D., Simmons**, **M.J.** (2000). Principles of genetic, 2nd Edition New York;

Solomon, E.P., Berg, L.R., Martin, D.W. (1999). Biology, 5th edition, Sounders Collage Publishing, USA.

Sonderegger, M., and Sauer, U., (2003). Evolutionary Engineering of *Saccharomyces cerevisiae* for Anaerobic Growth on Xylose, *Applied and Environmental Microbiology*, 69, 1990–1998.

Takano, J., Kobayashi, M., Noda, Y., Fujiwara, T. (2007). *Saccharomyces cerevisiae* Bor1p is a boron exporterand a key determinant of boron tolerance, *FEMS Microbiol Lett*, 267, 230–235.

Takano, J., Miwa, K., Fujiwara, T. (2008). Boron transport mechanisms: collaboration of channels and transporters. Trends Plant Sci 13: 451-457.

Takano, J., Miwa, K., Yuan, L., von Wiren, N. and Fujiwara, T. (2005). Endocytosis and degradation of BOR1, a boron transporter of Arabidopsis thaliana, regulated by boron availability. Proc. Nat Acad. Sci. USA 102: 12276–12281.

Takano, J., Noguchi, K., Yasumori, M., Kobayashi, M., Gajdos, Z., Miwa, K., Hayashi, H., Yoneyama, T., Fujiwara, T., (2002). Arabidopsis boron transporter for xylem loading. Nature 420: 337–340.

Tanaka, M., and Fujiwara, T. (2008). Physiological roles and transport mechanisms of boron: perspectives from plants. Pflu¨gers Arch. Eur. J. Physiol. 456: 671–677.

Uluisik, I., Kaya, A., Fomenko, D.E., Karakaya, H.C., Carlson, B.A., Gladyshev, V.N., Koc, A. (2011). Boron Stress Activates the General Amino Acid Control Mechanism and Inhibits Protein Synthesis. PLoS ONE; Volume 6, Issue 11, e27772.

Warington, K. (1923). The effect of boric acid and borax on the broad been and certain other plants, *Ann. Bot*. 37, 629–672.

Wheals, A. (2003). **Scanning electron micrograph of** *S. cerevisiae***.** University of Bath, UK.

Wisselink, H. W., Toirkens, M. J., del Rosario Franco Berriel, M., Winkler, A. A., van Dijken, J. P., Pronk, J. T., van Maris, A. J. (2007). Engineering of Saccharomyces cerevisiae for efficient anaerobic alcoholic fermentation of L-arabinose. *Appl Environ Microbiol, 73*(15), 4881-4891.

Url-1 http://mips.helmholtz-muenchen.de/proj/funcatDB/, date retrieved 10.04.2013.

Url-2 http://funspec.med.utoronto.ca/, date retrieved 17.04.13.

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Musa Tartik, Ülkü Yılmaz, Melike Özgül, Özge Özmeral, Z. Petek Çakar, Molecular characterization of a boron-resistant yeast mutant obtained by evolutionary engineering, Firsth International Molecular Biyology and Genetic Congress, 2012 November 23-24

Musa Tartik, Ülkü Yılmaz, Ceren Alkım, Z. Petek Çakar; Whole genomic analysis of a boron-resistant yeast mutant obtained by evolutionary engineering, Fifth Conference on Physiology of Yeast and Filamentous Fungi June 4 - 7, 2013, Montpellier, France